Leena Erkkilä

PULMONARY INFECTION AND ATHEROSCLEROSIS IN AN EXPERIMENTAL *CHLAMYDIA PNEUMONIAE* MODEL

ACADEMIC DISSERTATION

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National Public Health Institute, Department of Viral Diseases and Immunology, Laboratory for Chlamydia and Respiratory Tract Bacteria, Oulu, Finland

and

University of Helsinki, Faculty of Biosciences, Department of Biological and Environmental Sciences, Division of General Microbiology, Helsinki, Finland

and

University of Oulu, Faculty of Medicine, Department of Medical Microbiology, Oulu, Finland

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Kansanterveyslaitos (KTL)
Mannerheimintie 166
00300 Helsinki
Puh. vaihde (09) 474 41, telefax (09) 4744 8408

Folkhälsoinstitutet
Mannerheimvägen 166
00300 Helsingfors
Tel. växel (09) 474 41, telefax (09) 4744 8408

National Public Health Institute
Mannerheimintie 166
FIN-00300 Helsinki, Finland
Telephone +358 9 474 41, telefax +358 9 4744 8408

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Supervised by

Research Professor Maija Leinonen, PhD
Department of Viral Diseases and Immunology
National Public Health Institute
Oulu, Finland

Docent Kirsi Laitinen, PhD
Department of Public Health
University of Helsinki
Helsinki, Finland

Emeritus Professor Pekka Saikku, MD, PhD
Department of Medical Microbiology
University of Oulu
Oulu, Finland

Reviewed by

Professor Petri Kovanen, MD, PhD
Wihuri Research Institute
Helsinki, Finland

and

Professor Matthias Maass, MD
Institute of Medical Microbiology
University of Lübeck
Lübeck, Germany

Opponent

Professor Gerald I. Byrne, PhD
Department of Molecular Sciences
University of Tennessee Health Sciences Center
Memphis, USA

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ABSTRACT

Chlamydia pneumoniae is a Gram-negative bacterium, which has a unique intracellular developmental cycle for replication inside host cells. *C. pneumoniae* infects the respiratory tract and is a common cause of upper respiratory tract infections as well as bronchitis and atypical pneumonia, which may be mild or life-threatening. Similarly to all chlamydial species, *C. pneumoniae* has a tendency to cause chronic infections, which may lead to severe disease sequelae, such as chronic pulmonary diseases and atherosclerosis. In an experimental *C. pneumoniae* infection model, *C. pneumoniae* and chronic inflammation persist in mouse lung. Among the individual chlamydial antigens, chlamydial Heat shock protein 60 (cHsp60) is associated with chronic diseases. The immune response against cHsp60 and, further, the development of autoimmunity against host Hsp60 are associated with a chronic inflammatory disease, namely atherosclerosis. In the prevention of cardiovascular diseases, lipid-lowering statin therapies are widely used. They inhibit the rate-limiting step in the mevalonate pathway and cholesterol biosynthesis and have been shown to have antimicrobial effects.

In the present study, a mouse model for *C. pneumoniae* infection was used. Specifically, the technical aspects, the pulmonary infection, the development of atherosclerotic lesions and Hsp60 autoimmunity, and the possible effect of simvastatin treatment on *C. pneumoniae* infection were studied. Mice were infected with *C. pneumoniae* and, in some experiments, fed a diet with some cholesterol supplementation or a high-fat diet.

One of the most important findings of the present study was that the host cell components in the *C. pneumoniae* inoculum may induce a weak inflammatory response. This may affect the course of infection by, for instance, reactivating a culture-negative infection. It was also shown that chronic *C. pneumoniae* infection led to the development of a humoral Hsp60 autoimmune response along with the development of initial aortic atherosclerotic lesions. The Hsp60 autoimmune response developed after repeated *C. pneumoniae* exposures and was exaggerated if the mice were also fed a cholesterol-supplemented diet. In addition, treatment with simvastatin was shown to exhibit an antichlamydial effect *in vivo*. The effect was seen as diminished chlamydial growth in the lungs.

Keywords: *Chlamydia pneumoniae; Chlamydophila pneumoniae;* Mice; Models, animal; Disease models, animal; Pneumonia; Pulmonary infection; Hsp60 autoimmune response; Chronic infection; Arteriosclerosis; Atherosclerosis; Simvastatin

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TIIVISTELMÄ

Chlamydia pneumoniae on Gram-negatiivinen bakteeri, jolla on solunsisäinen lisääntymiskierto. *C. pneumoniae* infektoi hengitysteitä ja on yleinen ylähengitystieinfetioiden, ja myöskin bronkiitin ja atyyppisen pneumonian aiheuttaja. Infektio voi olla lievä tai hengenvaarallinen. *C. pneumoniae*:lla kuten muillakin klamydialajeilla on taipumus aiheuttaa kroonisia infektioita, jotka voivat johtaa vakavimpiin jälkiseurauksiin, kuten kroonisiin keuhkotauteihin ja ateroskleroosiin. Kokeellisessa infektiomallissa, *C. pneumoniae* ja krooninen tulehdus persistoivat hiiren keuhkoissa. Yksi tärkeimmistä klamydian antigeeneistä, joka on yhdistetty kroonisiin tautimuotoihin, on klamydian 60kD:n stressiproteiini Hsp60. Sitä vastaan syntyvä immuunivaste ja mahdollinen autoimmuunivaste isännän omaa Hsp60:a vastaaan voivat osallistua kroonisen tulehdustaudin, ateroskleroosin kehittymiseen. Sydän- ja verisuonitautien ehkäisyssä ja hoidossa käytetään laajasti kolesterolia alentavia statiineja. Ne inhiboivat kynnysreaktiota mevalonaatti-synteesipolulla ja kolesterolin biosynteesissä ja niillä on havaittu olevan antimikrobisia vaikutuksia.

Tässä tutkimuksessa käytettiin hiirimallia *C. pneumoniae* infektiolle. Tutkimuksen kohteena olivat hiirimallin tekninen suorittaminen, keuhkoinfektio ja siihen vaikuttavat tekijät, ateroskleroosin ja autoimmuunivasteen kehittyminen Hsp60:lle, sekä kolesterolilääke simvastatiinin mahdollinen vaikutus *C. pneumoniae* infektioon. Hiiret infektoitiin nenän kautta *C. pneumoniae*:lla, ja joissakin kokeissa käytettiin rehua, jossa oli ylimääräinen annos kolesterolia tai rasvaa.

Tutkimuksen yksi tärkeimmistä tuloksista oli, että infektoinnissa käytettävän *C. pneumoniae* -preparaatin sisältämät isäntäsolujen rippeet saattavat aiheuttaa lievää tulehdusreaktiota. Tämä voi vaikuttaa infektion kulkuun mm. reaktivoimalla piilevän infektion. Lisäksi osoitettiin, että krooninen *C. pneumoniae* infektio johtaa Hsp60-autovasta-aineiden lisääntymiseen, sekä alkuvaiheessa olevien ateroskleroottisten plakkien kasvamiseen. Hsp60 autoimmuunivaste kehittyi toistuvien *C. pneumoniae* infektioiden myötä, ja voimistui mikäli hiiriä syötettiin rehulla, jossa oli kolesterolilisä. Simvastatiini-hoidolla havaittiin lisäksi olevan klamydian kasvua hillitsevä vaikutus, joka näkyi vähentyneenä klamydian lisääntymisenä hiiren keuhkoissa.

Avainsanat: *Chlamydia pneumoniae;* Keuhkoklamydia; Hiirimalli; Keuhkokuume; Keuhkoinfektio; Hsp60 autoimmuunivaste; Krooninen infektio; Ateroskleroosi; Simvastatiini

Contents

Abbreviations

AAA	abdominal aortic aneurysm
AM	alveolar macrophage
ApoE-KO	Apolipoprotein E knockout
bp	base pair
CAD	coronary artery disease
CAP	community-acquired pneumonia
cHsp60	chlamydial heat shock protein 60
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
DMSO	dimethylsulfoxide
EB	elementary body
EIA	enzyme immunoassay
HDL	high-density lipoprotein
hHsp60	human heat shock protein 60
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
Hsp	heat shock protein
i.c.	intracerebral
IFN-γ	interferon -γ
IFU	inclusion-forming unit
Ig	immunoglobulin
i.g.	intragastric
IL	interleukin
i.n.	intranasal
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
i.v.	intravenous
K6	Kajaani 6, *Chlamydia pneumoniae* isolate
K7	Kajaani 7, *Chlamydia pneumoniae* isolate
LBP	lipopolysaccharide-binding protein
LDL	low-density lipoprotein

LDLR-KO	low-density lipoprotein receptor knockout
LPS	lipopolysaccharide
mbHsp65	mycobacterial heat shock protein 65
MBL	mannose-binding lectin
MCP-1	monocyte chemoattractant protein-1
mHsp60	mouse Hsp60
MOMP	major outer membrane protein
Nod	nucleotide-binding oligomerization domain
OmpA	outer membrane protein A
OVA	ovalbumin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
p.i.	postinfection
PMN	polymorphonuclear
Pmp	polymorphic membrane protein
RB	reticulate body
rRNA	ribosomal RNA
SAA	serum amyloid A
SPG	sucrose-phosphate-glutamate
Th	T-helper
TLR	toll-like receptor
TNF-α	tumour necrosis factor-α
TW-183	*Chlamydia pneumoniae* isolate
TWAR	synonym for *Chlamydia pneumoniae*

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. These articles are reproduced with the kind permission of their copyright holders.

I Erkkilä L, Rottenberg ME, Laitinen K (2000) Comparison of anesthetics for inoculation of mice with *Chlamydia pneumoniae*. Comp Med 50:46-48

II Erkkilä L, Laitinen K, Laurila A, Saikku P, Leinonen M (2002) Experimental *Chlamydia pneumoniae* infection in NIH/S mice: effect of reinoculation with chlamydial or cell preparation on culture, PCR and histological findings of the lung tissue. Vaccine 20:2318-2324

III Erkkilä L, Laitinen K, Haasio K, Tiirola T, Jauhiainen M, Lehr HA, Aalto-Setälä K, Saikku P, Leinonen M (2004) Heat shock protein 60 autoimmunity and early lipid lesions in cholesterol-fed C57BL/6JBom mice during *Chlamydia pneumoniae* infection. Atherosclerosis 177:321-328

IV Erkkilä L, Jauhiainen M, Laitinen K, Haasio K, Tiirola T, Saikku P, Leinonen M (2005) Effect of simvastatin, an established lipid-lowering drug, on pulmonary *Chlamydia pneumoniae* infection in mice. Antimicrob Agents Chemother 49:3959-3962.

In addition, some unpublished data are presented.

1 Review of the literature

1.1 Bacterial pathogens and the respiratory tract

The human respiratory tract is divided into the upper and lower airways. The ascending bronchi narrow through dichotomous branching, and only particles smaller than 10 µm in diameter are able to reach the lung along with the airflow, and particles smaller than 3-5 µm have direct access into the bronchioles.

The bacteria causing respiratory infections may be part of the normal flora in the upper respiratory tract or may be acquired from the environment within aerosols or droplets. In the respiratory tract, bacteria are in contact with the ciliated epithelium, which attempts to push the trapped particles upward within mucus. The alveoli are lined by a thin layer of alveolar lining fluid, which contains soluble immune molecules, complement, collectins (e.g. surfactants A and D), and immunoglobulins. Soluble factors and cells of the innate immune system (lymphocyte-independent immunity) serve as the first-line defence against invading microbes. Dendritic cells are present in the submucosa of the nasopharynx, trachea and bronchial tree, and alveolar macrophages (AM) within the alveoli, with approximately one AM per alveolus. In addition to the immune cells in tissues, those residing in blood, i.e. monocytes and phagocytic neutrophils, are rapidly extravasated from blood when needed in defence against pulmonary infection. Blood-derived monocytes differentiate into macrophages in tissues. Monocyte and neutrophil cytokine signalling activates other immune cells. Cytokine signalling also induces the secretion of acute phase proteins as C-reactive protein (CRP). For innate immune recognition and subsequent phagocytosis and/or pro- or anti-inflammatory activation, pre-exposure is not needed, but molecular detection is based on structural divergence between procaryotes and eucaryotes. Procaryotes have conserved motifs, called pathogen-associated molecular pattern, e.g. lipopolysaccharide (LPS) and lipoteichoid acid. Immune cells recognize these through pattern recognition receptors, which are surface or cytosolic molecules, e.g. CD14, toll-like receptors (TLR), cytosolic nucleotide-binding oligomerization domain (Nod) receptors, LPS-binding protein (LBP), and mannose-

binding lectin (MBL) (Aderem and Ulevitch, 2000; Gordon and Read, 2002). Innate immune responses activate lymphocyte-dependent immunity, i.e. T- and B-cell responses.

If a pathogen is able to reach the alveoli and remains alive, it is widely dispersed onto the epithelium and may disseminate from one alveolus to another through, for instance, alveolar pores (pores of Kohn). Further, though there are effective anti-infective mechanisms to protect the organism against airborne pathogens entering the circulating blood in gas-exchanging areas, common bacteria causing pneumonia can also be detected in circulation.

1.2 *C. pneumoniae* diseases of the respiratory tract

1.2.1 Acute respiratory diseases

The outcome in host-microbe interaction, in general, is influenced by microbial and host-related factors. The outcome and possible damage vary between different microbes and hosts and between a certain microbe and its host over time, and the process keeps changing continuously (Fig. 1). (Casadevall and Pirofski, 1999). In commensalism and symbiosis, no damage to the host occurs, and in symbiosis, both benefit from the interaction. Colonization is a continuum of damage, which may progress to disease or persistence. In persistence, the host suffers continuous damage, and microbial persistence may be reactivated into overt disease. (Casadevall and Pirofski, 2000).

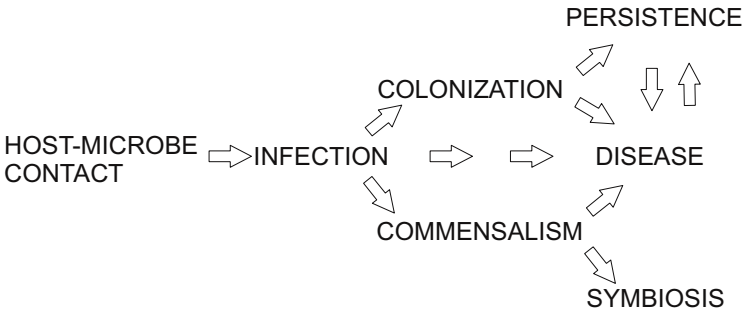


Fig. 1. Outcome of host-microbe interaction in infection. If the microbe is not eradicated, infection may result in colonization, disease, or commensalism. Colonization may further develop into persistence (chronicity/latency) and/or disease, and commensalism into symbiosis or disease if sufficient damage ensues from the interaction. Modified from (Casadevall and Pirofski, 2000).

C. pneumoniae, which spreads through the respiratory tract, is transmitted from person to person relatively inefficiently (Kleemola et al., 1988; Grayston et al., 1990; Aldous et al., 1992; Grayston et al., 1993), but it is still assumed that almost all people get infected with it at least once during their lifetime. Adults have 60-70 % seroprevalence, and men have higher seroprevalence than women (Grayston, 1992). The outcome of *C. pneumoniae*

infection in the respiratory tract may vary from asymptomatic nasopharyngeal carriage (Hammerschlag, 2004) through mild upper respiratory tract infections, sinusitis, pharyngitis, and otitis media to lower respiratory tract infections such as bronchitis (Grayston, 1992) and life-threatening pneumonia (Troy et al., 1997), and even severe systemic diseases have been reported (Grayston, 1994). As the high prevalence rates suggest, most (70 %) *C. pneumoniae* infections remain asymptomatic or mild, while a smaller proportion (30 %) result in more severe diseases (Hahn et al., 2002). Although some typical features exist, e.g. prolonged cough, (Miyashita et al., 2003) and some patient groups may be more likely to have *C. pneumoniae* disease than others, there are no specific clinical findings associated with *C. pneumoniae* respiratory infections (Grayston, 1992). One study showed that the highest predictive values for *C. pneumoniae* etiology was found in patients from nursing homes, with low CRP levels, non-productive cough, and normal urine analysis (Socan et al., 2004). In industrialized countries, community-acquired pneumonia (CAP) is the most common serious infectious disease. In Finland, the most common etiological agent of bacterial pneumonia, *Streptococcus pneumoniae*, causes 40-50 % of all CAP cases, while atypical bacteria, *Mycoplasma pneumoniae* and *C. pneumoniae,* cause 10-20 % of pneumonia cases during interepidemic periods (Jokinen et al., 2001). *C. pneumoniae* is evidently not an important cause of severe infections in small children (Chirgwin et al., 1991; Grayston, 1994), though severe pulmonary involvement has been reported to occur in otherwise healthy children and in adults (Hammerschlag, 2004), but most commonly, it causes mild pneumonias in school-aged children and healthy adolescents, e.g. college students and military conscripts (Kleemola et al., 1988; Grayston et al., 1993).

In *C. pneumoniae* disease, the damage is both pathogen- and host-mediated, and immune defence evidently plays a significant role in directing the respiratory outcome and cardiovascular dissemination. *C. pneumoniae* reinfections or recurrent infections may occur at short intervals. In fact, at least part of recurrent infections may actually be reactivations of chronic infections (Kleemola et al., 1988; Ekman et al., 1993). *C. pneumoniae* may also persist in the lungs of subjects with normal lung funtion (Wu et al., 2000; Kotsimbos et al., 2005). Chronic quiescent infection is possibly reactivated when appropriate, e.g. immunosuppressive, conditions emerge, as has been reported during hospitalization or in experimental animals on immunosuppressive cortisone treatment (Grayston et al., 1989a; Malinverni et al., 1995a; Laitinen et al., 1996).

1.2.2 Chronic pulmonary diseases

Evidently, persistent *C. pneumoniae* infection in the lungs contributes to several chronic pulmonary diseases (Laurila et al., 1997b), asthma and chronic obstructive pulmonary disease (COPD) being the most intensively studied. Adult-onset asthma, wheezing, asthmatic bronchitis, more severe asthma, and in asthmatic children, acute wheezing episodes and frequent exacerbations may be *C. pneumoniae*-associated (Hahn et al., 1991; Emre et al., 1994; Hahn et al., 1996; Cunningham et al., 1998; Black et al., 2000). Polymorphism of the host MBL gene seems to affect the association between *C. pneumoniae* and asthma (Nagy et al., 2003). COPD and its acute exacerbations may also be *C. pneumo-*

niae-associated (Blasi et al., 1993; von Hertzen et al., 1995; von Hertzen et al., 1996; von Hertzen et al., 1997; Wu et al., 2000). Other chronic lung processes whose serological associations with *C. pneumoniae* infection have been studied include sarcoidosis (Puolakkainen et al., 1996) and lung cancer (Laurila et al., 1997a; Jackson et al., 2000; Littman et al., 2005).

1.3 C. pneumoniae

1.3.1 History and taxonomy

C. pneumoniae was first found to be a human pathogen during mild pneumonia epidemics in Finland, as demonstrated by the antibody response against the first *C. pneumoniae* isolate TW-183 (Saikku et al., 1985). Later, respiratory tract–derived isolates were obtained from patients during acute respiratory tract infection (Grayston et al., 1986). The two first isolates (TW-183 and AR-39) were originally given the strain name TWAR (Grayston et al., 1986). They were serologically unique, and less pathogenic in mice than the other *C. psittaci* and *C. trachomatis* strains. Based on their morphology and staining with iodine (*C. psittaci* inclusions do not stain with iodine contrary to *C. trachomatis* inclusions), however, they were considered as *C. psittaci* (Kuo et al., 1986). Eventually, in 1989, the strain TWAR was classified as a new species based on elementary body (EB) ultrastructure (Chi et al., 1987), DNA analysis (Campbell et al., 1987; Cox et al., 1988), and serology (Kuo et al., 1986) and given the name *C. pneumoniae* (Grayston et al., 1989b).

In the order *Chlamydiales*, only the family *Chlamydiaceae* and its pathogenic *Chlamydia* species - *C. pneumoniae*, *C. psittaci* and *C. trachomatis* - were identified. Recently, environmental species with similar characteristics - obligate intracellular growth and unique developmental cycle - belonging to three more families, *Parachlamydiaceae*, *Simkaniaceae* and *Waddliaceae*, have been recognized, and the possibility of emerging pathogens within these families has been recognized (Amann et al., 1997; Kahane et al., 1998; Everett et al., 1999; Rurangirwa et al., 1999; Friedman et al., 2003). Environmental chlamydiae may have twice as large genomes as pathogenic chlamydiae, and although there is little evidence of recent lateral gene acquisition, they contain the same virulence factors as pathogenic chlamydiae (Horn et al., 2004). In 1999, based on 16S and 23S ribosomal RNA (rRNA) sequences, the family *Chlamydiaceae*, which contains the pathogenic chlamydiae, was taxonomically reclassified (Everett et al., 1999), and one genus in the former, namely *Chlamydia*, was divided into two genera: *Chlamydia* and *Chlamydophila* (Fig. 2). *C. pneumoniae* was then renamed as belonging to the genus *Chlamydophila*. However, chlamydiae are still considered to constitute a unique group of bacteria that should not yet be separated (Schachter et al., 2001), and the original name *Chlamydia pneumoniae* is used alongside the proposed new one, and actually even more frequently (PubMed, year 2004, *Chlamydia pneumoniae* or *Chlamydophila pneumoniae* as a title/abstract word: 238 vs. 42, respectively). In addition, the new classification also iden-

tified the animal pathogen species in the *Chlamydiaceae* family (Fig. 2). Recently, the previous belief that *C. pneumoniae* is only a human pathogen, has changed. As a possible disease reservoir, *C. pneumoniae* infections do occur in wild and domestic animals. Natural hosts include horse, koala and amphibians (reviewed in (Longbottom and Coulter, 2003)) as well as reptiles (Bodetti et al., 2002) and dogs (Sako et al., 2002). Varying clinical signs, including atherosclerosis, have been reported in these animals, too.

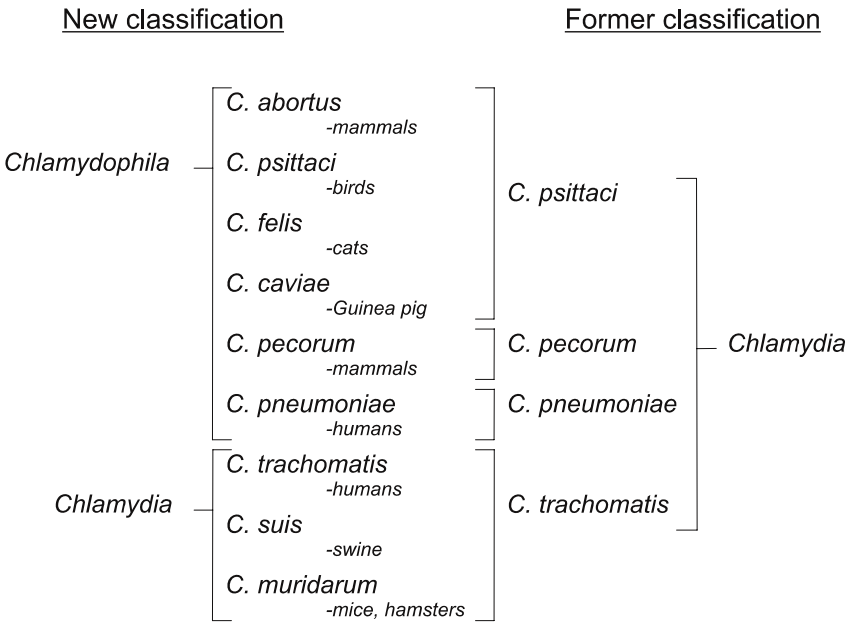
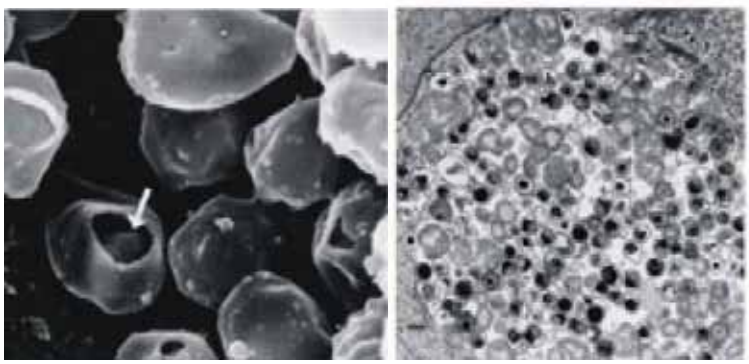


Fig. 2. New and former classification of *Chlamydiaceae*. The most common hosts for each species are listed. For details concerning the genus name of *C. pneumoniae*, see the text. Modified from (Bush and Everett, 2001).

1.3.2 Cell biology

The complete *C. pneumoniae* genome size is 1.23×10^6 base pairs (bp) (Kalman et al., 1999), and the interstrain identity between the thus far sequenced strains (AR-39, J138, CWL029 and TW-183) is >99.9% (Read et al., 2000a; Shirai et al., 2000). The chlamydial nucleoid consists of the chromosome and of two eucaryotic histone-like proteins (Hackstadt et al., 1991). Some biovars may contain a plasmid (Everett et al., 1999), and a 4524-nucleotide single-stranded DNA bacteriophage (φCpn1) has been found in the strain AR39 but not in the other strains studied (Read et al., 2000a; Read et al., 2000b). Chlamydiae are strictly intracellular bacteria that multiply within inclusions in host cells and avoid lysosomal fusion (Moulder, 1991; Al-Younes et al., 1999). Chlamydial particles are non-motile and have a large periplasmic space (Fig. 3.a.). The pointed end of the pear-shaped EB particle may be the first part to contact the host cell (Kuo et al., 1988). The cell wall of *C. pneumoniae* resembles that of gram-negative bacteria, and the outer

membrane contains LPS. Chlamydiae have genes for peptidoglycan synthesis, but the issue of whether peptidoglycan exists is controversial (Ghuysen and Goffin, 1999). Systematic genome studies of *C. pneumoniae* surface proteins (outer membrane, periplasmic and inner membrane proteins) have identified 53 proteins, which include a major outer membrane protein (MOMP)/outer membrane protein A (OmpA), PorB, a 76-kDa protein homolog, 11 proteins of the polymorphic membrane protein (Pmp) family, a cysteine-rich 60-kDa protein encoded by *omcB* (Omp3), a 9-kDa cysteine-rich outer membrane complex lipoprotein OmcA (Omp2), DnaK and Mip-like proteins, hypothetical protein, and type-three secretion system proteins (Montigiani et al., 2002), which may provide a direct connection between chlamydial particles and the host cell cytoplasm (Miyashita et al., 1993). Several molecules are involved in the attachment and subsequent endocytosis of chlamydiae, e.g. the host cell's heparan sulfate-like glycosaminoglycan (Wuppermann et al., 2001), and the cholesterol-containing lipid rafts on the host cell plasma membrane (Stuart et al., 2003). Once internalized within an inclusion, *C. pneumoniae* EBs reorganize into vegetative reticulate bodies (RBs), which multiply by binary fission during their logarithmic growth and occasionally possibly by budding (Wolf et al., 2000) (Fig. 3.b.). At the end of the cycle, RBs reorganize back into more condensed and rigid EBs, and oxidation of the outer membrane cysteine-rich proteins takes place. Both nonlytic and lytic release of infectious progeny occurs (Gieffers et al., 2002). The ATP required for *C. pneumoniae* growth is derived both from the host cell (ATP/ADP translocase) and from chlamydial *de novo* synthesis (Read et al., 2000a).



A B

Fig. 3. A) Scanning electron micrograph of *C. pneumoniae* AR-39 EBs inside an inclusion in a HeLa cell. The arrow points to the large periplasmic space between the outer membrane and the cytoplasmic membrane of EB at the breaking point. B) Transmission electron micrograph of C. pneumoniae AR-39 inclusion in a HeLa cell 60 hours after infection. Both RBs and EBs are present. (Wolf et al., 2000).

1.3.3 Experimental in vivo *models for* C. pneumoniae *infection*

Mouse is most commonly used in experimental *C. pneumoniae in vivo* studies (Tables 1 and 2). In the first reported study, Swiss Webster mice were infected intranasally (i.n.), intrave-

nously (i.v.), or intracerebrally (i.c.), and the organism was recovered from different tissues: from the brain after an i.c. challenge, from the lungs after an i.n. challenge, and from the spleen, liver, and lungs after an i.v. challenge. In the same tissues, inflammatory reactions were detected by histology (Kuo et al., 1986). The course of mouse lung infection after i.n. inoculation during primary and reinfection was later described in more detail (Kaukoranta-Tolvanen et al., 1993; Yang et al., 1993), and i.n. infection became the most commonly used route of challenge in lung infections as well as later in atherosclerosis models. In primary infection, culture positivity in the lungs lasts for a few weeks. Inflammation, as detected histologically, persists in the lungs beyond the culture-positive period. After reinfection, the culture-positive period is shorter, and the inflammatory reaction is exaggerated. Variations occur between different mouse strains, *C. pneumoniae* isolates, and challenging doses. The use of more sensitive methods, such as polymerase chain reaction (PCR), in detecting *C. pneumoniae* in animal tissues has extended the *C. pneumoniae*-positive period to several months after the last challenge. Clinical signs in mice and other species are usually scarce, unless a high inoculation dose is given.

Table 1. C. pneumoniae *studies in mouse models.*

Study topic	Reference	Infection route	Cpn strain	Mouse strain
Infection course	(Kuo et al., 1986)	i.n., i.c., i.v.	AR-39	Swiss Webster
			TW-183	
	(Kishimoto, 1990)	i.n.	TW-183	ICR
	(Kaukoranta-Tolvanen et al., 1993)	i.n.	TW-183	Swiss Webster
			K6	NIH/S
			Helsinki 12	BALB/c
	(Yang et al., 1993)	i.n.	AR-39	Swiss Webster
				ICR
				BALB/cAnN
				C57BL/6N
				B6C3F1
				C3H/HeN
	(Kimura, 1994)	i.n.	TW-183	IHC
	(Yang et al., 1994)	i.n.	AR-39	Swiss Webster
	(Kaukoranta-Tolvanen et al., 1995)	i.n.	K6	NIH/S
	(Malinverni et al., 1995a)	i.n.	AR-39	Swiss Webster
	(Yang et al., 1995)	i.n., i.v., s.c.	AR-39	Swiss Webster
	(Laitinen et al., 1996)	i.n.	K6	NIH/S
	(Moazed et al., 1998)	i.p.	AR-39	C57BL/6J
	(Erkkilä et al., 2002)	i.n.	K6	NIH/S
	(May et al., 2003)	i.v.	CM1	C57BL/6J
	(Shi et al., 2003)	i.n., i.v.	CWL-029	Icr
	(Little et al., 2005)	i.n.	AR-39	BALB/c

Table 1. Continued

Technique	(Erkkilä et al., 2000)	i.n.	K6	NIH/S
			K7	C57BL/6J
Atherosclerosis	(Moazed et al., 1997)	i.n.	AR-39	ApoE-KO
				C57BL/6J
	(Hu et al., 1999)	i.n.	AR-39	LDLR-KO
	(Moazed et al., 1999)	i.n.	AR-39	ApoE-KO
	(Blessing et al., 2000)	i.n.	AR-39	C57BL/6J
	(Liu et al., 2000)	i.n.	AR-39	LDLR-KO
	(Liuba et al., 2000)	i.n.	IOL 207	ApoE-KO
	(Aalto-Setälä et al., 2001)	i.n.	K7	ApoE-KO
	(Blessing et al., 2001)	i.n.	AR-39	C57BL/6J
	(Burian et al., 2001a)	i.n.	TWAR	BALB/c
	(Burnett et al., 2001)	i.n.	na	ApoE-KO
	(Caligiuri et al., 2001)	i.n.	na	C57BL/6J
				ApoE-KO
	(Rothstein et al., 2001)	i.n.	A-03	ApoE-KO
	(Blessing et al., 2002a)	i.n.	AR-39	C57BL/6J
	(Ezzahiri et al., 2002)	i.p.	TWAR2043	ApoE3-Leiden
	(Chesebro et al., 2003)	i.n.	AR-39	eNOS-/-
				iNOS-/-
	(Ezzahiri et al., 2003)	i.p.	TWAR2043	LDLr/ApoE-/-
	(Erkkilä et al., 2004)	i.n.	K7	C57BL/6JBom
	(Sharma et al., 2004)	i.n.	AR-39	LDLR-KO
Lipids	(Tiirola et al., 2002)	i.n.	K7	NIH/S
Alzheimer	(Little et al., 2004)	i.n.	96-41	BALB/c
Immunology	(Peterson et al., 1996)	i.p.	TW-183	BALB/c
				C57BL/6
	(Penttilä et al., 1998b)	i.n.	K6	NIH/S
				BALB/c
				C57BL/6J
	(Penttilä et al., 1998a)	i.n.	K6	BALB/c
	(Peterson et al., 1998)	i.n.	TW-183	Swiss Webster
			2043	
			CM-1	
	(Penttilä et al., 1999)	i.n.	K6	BALB/c
				nu/nu
	(Rottenberg et al., 1999)	i.n.	K6	C57BL/6
				BALB/c
				multiple KOs
	(Geng et al., 2000a)	i.n.	TW-183	BALB/c
				129
				G129
	(Murdin et al., 2000)	i.n.	AR-39	BALB/c
	(Pal et al., 2000)	i.n.	TW-183	129sv
				Nramp1d
	(Penttilä et al., 2000)	i.n.	K6	BALB/c
	(Rottenberg et al., 2000)	i.n.	K6	C57BL/6
				multiple KOs
	(Svanholm et al., 2000)	i.n.	K	C57BL/6
				multiple KOs

Table 1. Continued

	(Vuola et al., 2000)	i.n.	K6	BALB/c C57BL/6JBom
	(Bandholtz et al., 2002)	i.n.	K6	C57BL/6 multiple KOs
	(Airaksinen et al., 2003)	i.n.	K6	BALB/c
	(Burian et al., 2003)	i.n.	TW-183	BALB/c +KO
	(Mueller et al., 2004)	i.n.	HK	Swiss Webster C3H/HeN C3H/HeJ TLR2+/+, -/-
	(Mygind et al., 2004)	i.n.	na	C57BL/6J
	(Penttilä et al., 2004)	i.n.	K6	BALB/c
	(Rothfuchs et al., 2004)	i.n.	K6	C57BL/6 multiple KOs
Antibiotics	(Nakata et al., 1994)	i.n.	K6 IOL 207	MHC-ICR
	(Malinverni et al., 1995b)	i.n.	AR-39	Swiss Webster
	(Masson et al., 1995)	i.n.	TW-183	NIH/S BALB/c MF1 C3H/He
	(Wolf and Malinverni, 1999)	i.n.	AR-39	NMRI
	(Bin et al., 2000)	i.n.	AR-39	NMRI
	(Törmäkangas et al., 2004b)	i.n.	K7	C57BL/6J
	(Törmäkangas et al., 2004a)	i.n.	K7	C57BL/6J
	(Blessing et al., 2005)	i.n.	AR-39	ApoE-KO

Abbreviations used in this table: i.n.=intranasal; i.c.=intracranial; i.v.=intravenous; s.c.=subcutaneous; i.p.=intraperitoneal; Cpn=C. pneumoniae; na=not assessed; ApoE-KO=ApoE knockout; LDLR-KO=LDL receptor knockout.

Table 2. Other C. pneumoniae *animal model studies.*

Animal species	Reference	Infection route	Cpn strain	Study topic
Monkey	(Kuo et al., 1986)	conjunctiva	TW-183	Infection course
	(Holland et al., 1990)	several mucosal sites	TW-183	Infection course
Baboon	(Bell et al., 1989)	i.t., several mucosal sites	AR-39	Infection course
Rabbit	(Moazed et al., 1996)	i.n., i.t.	AR-39	Atherosclerosis
	(Fong et al., 1997)	nasopharynx	VR1310	Atherosclerosis
	(Laitinen et al., 1997)	i.n.	K7	Atherosclerosis
	(Muhlestein et al., 1998)	i.n.	VR1310	Atherosclerosis
	(Fong et al., 1999a)	i.n.	AR-39	Atherosclerosis
	(Fong et al., 1999b)	i.n.	VR1310	Atherosclerosis
	(Tambiah et al., 2001)	laparotomy	na	AAA
	(Coombes et al., 2002)	i.n.	na	Atherosclerosis
	(Tambiah and Powell, 2002)	laparotomy	na	AAA
	(Gieffers et al., 2004b)	i.t.	CV-6	Infection course
Pig	(Liuba et al., 2003b)	i.t.	IOL-207	Atherosclerosis
	(Pislaru et al., 2003)	i.c., i.b.	AR-39	Atherosclerosis
Rat	(Herrera et al., 2003)	i.n.	AR-39	Atherosclerosis
Hamster	(Sambri et al., 2004)	i.p., i.n.	FB/96	Infection course
	(Finco et al., 2005)	i.p.	FB/96	Immunology

Abbreviations used in this table: i.t.=intratracheal; i.n.=intranasal; i.c.=intracoronary; i.b.=intrabronchial; na=not assessed; i.p.=intraperitoneal; Cpn=*C. pneumoniae*; AAA=abdominal aortic aneurysm.

1.4 Factors determining the persistence of *C. pneumoniae* infection in lung

1.4.1 Pulmonary inflammation

C. pneumoniae readily infects *in vitro* and *in vivo* cells from both the upper and the lower respiratory tract (Wong et al., 1992; Yang et al., 1994; Shemer-Avni and Lieberman, 1995; Alakärppä et al., 1999; Jahn et al., 2000; Meijer et al., 2000; Haralambieva et al., 2004). Deciliation of ciliated cells also occurs (Yang et al., 1994; Shemer-Avni and Lieberman, 1995). Attachment and invasion into epithelial cells activate directly e.g. nuclear factor-κβ activation/translocation and secretion of pro-inflammatory cytokines (IL-8), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), expression of leukocyte adhesion molecules, and subsequent enhanced polymorphonuclear (PMN) leukocyte transepithelial migration (Jahn et al., 2000; Yang et al., 2003). In mice, the expression of histidine decarboxylase, which generates histamine from histidine, is increased, especially in bronchial epithelial cells, which may have a deleterious role in *C. pneumoniae* infection

(Burian et al., 2003). The surfactant proteins A and D enhance the phagocytosis of *C. pneumoniae* (Oberley et al., 2004). In the alveoli, release of surfactant protein A by Type II pneumocytes may be inhibited during *C. pneumoniae* infection (Wissel et al., 2003).

The presence of inflammatory cells and inflammatory mediators in lungs reflect the local defense against invading *C. pneumoniae*. In mice, histology implies interstitial pneumonitis of varying severity during *C. pneumoniae* infection. The cellular picture during infection is of chronic type, i.e. it mainly consists of mononuclear cells, in peribronchial and perivascular infiltrations, although in the first few days PMN leukocytes may also infiltrate in large amounts into the bronchioles, alveolar space, and interstitium. Lymphoid follicles are also seen. (Kaukoranta-Tolvanen et al., 1993; Yang et al., 1993; Yang et al., 1994). In the *C. pneumoniae* mouse model, primary infection gives partial protection against reinfection. The role of various cell types in the overall outcome of infection or in the protection against reinfection has been studied, and T cells, both CD8+ and CD4+, were found protective (Penttilä et al., 1999; Rottenberg et al., 1999). *C. pneumoniae*-specific CD8+ have a T-helper 1 (Th1) type cytokine pattern, which is considered favorable for clearance, and they are able to lyse *C. pneumoniae*-infected cells. Multiple CD8+ cytolytic antigens/epitopes have been screened from the genome, some of which are protective (Saren et al., 2002; Wizel et al., 2002). The Th1 type also predominates against complete Pmps8, 20, and 21, and both CD8+ and CD4+ are needed for maximal responses (Mygind et al., 2004). Natural killer cell cytotoxicity does not seem to be necessary for controlling *C. pneumoniae* infection (Rottenberg et al., 2000).

The various protective effects of CD4+ cells, CD8+ cells, and macrophages seem to be mediated through IFN-γ secretion (Rothfuchs et al., 2004). IFN-γ also participates in the induction of e.g. tryptophan deprivation, inducible nitric oxide synthase (iNOS), iron deprivation, Th1/Th2 balance, and chemokine and adhesion molecule expression. In mouse pulmonary infection, IFN-γ restricts the growth of chlamydiae and histopathological changes and strengthens the Th1 type response (Penttilä et al., 1998a; Rottenberg et al., 1999; Vuola et al., 2000). Deleterious effects in *C. pneumoniae* infection and more severe disease are suggested to be mediated through delayed IFN-γ transcription and enhanced interleukin-4 (IL-4) and IL-10 transcription in lungs (shift from Th1 to Th2) (Rottenberg et al., 1999), high nitric oxide responders (Huang et al., 2002), and reduced iNOS transcription (Rottenberg et al., 1999).

1.4.2 Persistence of C. pneumoniae

Importantly, *C. pneumoniae* is able to resist immune defense and replicates and persists within immune cells. In addition to being able to replicate within phagocytes, macrophages, and neutrophils, *C. pneumoniae* replicates within nonphagocytic lymphocytes as well (Gaydos et al., 1996; Haranaga et al., 2001; van Zandbergen et al., 2004). Failure to clear the infection is due to several factors. The predominance of the Th2 type response in chlamydial diseases is usually associated with a failure in clearance (Holland et al., 1996). Chlamydiae may hamper the presentation of its antigens - chlamydial protease-like activity factor has been shown to degrade transcription factors and to suppress major histocompatibility complex class I and II expression (Zhong et al., 1999; Zhong et al.,

2000). Inhibition of apoptosis in several cell types, including immune cells, such as blood mononuclears and neutrophils (Geng et al., 2000b; Rajalingam et al., 2001; Airenne et al., 2002; van Zandbergen et al., 2004), may also predispose to chronicity. A certain cell type, type II alveolar epithelial cells (surfactant protein-A positive cells), has been found to be the host for *C. pneumoniae* in chronic lung disease (COPD) (Rupp et al., 2004a).

In vitro, chlamydial persistence is characterized by an altered developmental cycle (reviewed in (Beatty et al., 1994b)): development of aberrant forms and interruption in the development of infective EB forms. Transcription and translation are also altered in persistent chlamydial infection (Mathews et al., 2001; Molestina et al., 2002). In persistent *C. pneumoniae* infection, the expression of genes needed for DNA replication, repair, and chromosome segregation continues, but those encoding proteins for cell division are not involved (Byrne et al., 2001). ATP is probably derived from the host cell during persistence (Gerard et al., 2002). Importantly, persistent *in vitro* chlamydial infection induced by concentrations of IFN-γ not high enough to kill can be reactivated by anti-IFN-γ treatment or by replacing IFN-γ–containing medium with medium that does not contain IFN-γ (Shemer and Sarov, 1985; Beatty et al., 1993b; Mehta et al., 1998) through reorganization of persistent forms to infective EBs (Beatty et al., 1995). In experimental animals, *C. pneumoniae* infection can be reactivated with immunosuppressive treatment (Malinverni et al., 1995a; Laitinen et al., 1996). Concerning *C. pneumoniae in vitro*, spontaneous persistence takes place in blood mononuclear cells and alveolar macrophages (Airenne et al., 1999; Haranaga et al., 2003). In addition, persistence of *C. pneumoniae* can be induced by several agents *in vitro*, including IFN-γ alone or in combination with TNF-α (Summersgill et al., 1995), tobacco smoke (Wiedeman et al., 2004), and several antibiotics (Gieffers et al., 2004a).

1.4.3 Hsp60 in chlamydial persistence

In persisting chlamydial infections and associated chronic inflammation, the 60 kD Heat shock protein (Hsp), called chlamydial Hsp60 (cHsp60), is of special interest among the chlamydial antigens (Morrison et al., 1989; Toye et al., 1993). Hsps function as chaperones, and both microbial and host cells express large amounts of Hsps during infection. Both mammalian and microbial Hsps are able to induce activation of the immune system, and microbial Hsps are likely targets of the host immune response, because they may be processed along with other antigens and presented to macrophages, and they further contain conserved epitopes enabling interspecies cross-reaction (Young et al., 1988; Young and Elliott, 1989; Zügel and Kaufmann, 1999).

Chlamydiae have three Hsp60 genes (Stephens et al., 1998), and the proteins Hsp60 1-3 are encoded by the genes *groEL1 – 3* (Kalman et al., 1999). At the amino acid level, GroEL1 is the most conserved among the chlamydial species (91-98%), while GroEL2 and GroEL3 are conserved at a lower level (26-37%) (Karunakaran et al., 2003). There is a negative regulator protein HrcA, which binds to the CIRCE element found in *groEL1* containing *groE* operon (Wilson and Tan, 2002). Expression of Hsp60 (Hsp60 1) during actively replicating *C. trachomatis* infection *in vitro* occurs throughout the developmental cycle and may correlate with metabolic activity (Shaw et al., 2000). cHsp60 is detectable

inside a *C. pneumoniae* inclusion and in the culture supernatant, and it stimulates innate immune cells both *in vitro* and *in vivo* (da Costa et al., 2002; da Costa et al., 2004). During persistent IFN-γ–induced *C. trachomatis* infection, cHsp60 is found at almost normal levels compared to the low levels of, e.g., MOMP (Beatty et al., 1993a; Beatty et al., 1995). In *C. pneumoniae*, Hsp60/GroEL protein expression is also increased in persistent IFN-γ-induced infection (Molestina et al., 2002), and the Hsp60 expression of *C. pneumoniae* is similarly high in persistent alveolar macrophage infection (Haranaga et al., 2003) and in *ex vivo* COPD lung specimens (Rupp et al., 2004a). In persistent *C. trachomatis* serovar K infection, different transcription patterns were identified for the three different genes compared to active infection, and this pattern correlated with that of synovial tissue samples from *C. trachomatis* reactive arthritis patients (Gerard et al., 2004).

1.5 Association of atherosclerosis and *C. pneumoniae* infection

Although there are reports from as early as the mid-20th century focusing on the association of chlamydial (psittacosis and lymphogranuloma venereum) with arterial and cardiac diseases (Coutts and Davila, 1945; Sutton et al., 1967), the initial seroepidemiological study on the association of *C. pneumoniae* and atherosclerosis was published in 1988 (Saikku et al., 1988). Thereafter, other seroepidemiological studies as well as studies on the detection of *C. pneumoniae* in atherosclerotic lesions by electron microscopy, immunohistochemistry, PCR, and culture have been published (Shor et al., 1992; Kuo et al., 1993; Ramirez, 1996). Animal experiments revealed the development of atherosclerotic changes due to infection (Fong et al., 1997; Laitinen et al., 1997). Results from the first two small antibiotic prevention studies in humans were positive, but larger studies conducted thereafter have been controversial (Gupta et al., 1997; Gurfinkel et al., 1997; Wells et al., 2004; Anderson, 2005).

Chronic and relapsing *C. pneumoniae* infections are difficult to diagnose, which hampers association studies (Boman and Hammerschlag, 2002). In addition, studies on the effects of microbial and host gene polymorphism on the outcome of infection are just emerging. Yet, it is known that *C. pneumoniae* isolates vary in the copy number of the *tyrP* encoding an aromatic amino acid transporter - respiratory isolates have multiple copies, and cardiovascular isolates have only one copy of the gene (Gieffers et al., 2003). Diversity in *omp1* between the respiratory isolates and the coronary isolate has also been reported (Molestina et al., 1998). The presence of *C. pneumoniae* bacteriophage (φCpn1) has been associated with AAA, although φCpn1 does not contain any known virulence genes (Karunakaran et al., 2002). Certain host gene polymorphisms in the CD14 promoter with enhanced expression have been associated with *C. pneumoniae* infection (Heinemann et al., 1996; Eng et al., 2003) and, further, with chronic *C. pneumoniae* infection diagnosed by the presence of chlamydial DNA in peripheral blood monocytes of coronary artery disease (CAD) patients (Rupp et al., 2004b). In addition, IL-1 polymorphism has been linked with CAD in connection with *C. pneumoniae* infection (Momiyama et al., 2001), and *C. pneumoniae* seropositivity seems to be associated with high Lipoprotein(a) levels and certain human leukocyte antigen class II genotypes (Dahlen et al., 1995).

1.5.1 Inflammation in atherogenesis

Atherosclerosis is considered a low-grade chronic inflammatory disease of the arterial wall. The hallmark of the disease is lipid accumulation into the blood vessel walls. Lipids and inflammatory markers are present in circulating blood (reviewed in (Libby et al., 2002)). Slightly elevated levels of CRP have been shown to predict subsequent cardiovascular events (Libby and Ridker, 2004). The levels of CRP and other acute phase proteins increase to various degrees in several stress conditions, e.g. ongoing infection, inflammation, and trauma (Gabay and Kushner, 1999). During *C. pneumoniae* pneumonia, CRP levels are elevated, but remain lower than in, for instance, *S. pneumoniae* pneumonia or in coinfection by these two microbes (Kauppinen et al., 1996). Overall, the aggregate number of pathogens (pathogen burden) has been associated with the degree of inflammation, the extent of atherosclerosis, and the risk for death (Zhu et al., 2000; Zhu et al., 2001; Espinola-Klein et al., 2002a; Espinola-Klein et al., 2002b).

In the development of atherosclerotic lesions, endothelial dysfunction is the initial event, which leads to increased adhesion of leukocytes to the vascular wall and to inflammation. The earliest atherosclerotic lesion type, the fatty streak, is an inflammatory lesion consisting of T lymphocytes (both CD4+ and CD8+) and monocyte-derived macrophages. Foam cells are formed from macrophages in the presence of lipids. (Fig. 4) If the inflammatory process continues, more complicated lesions evolve, i.e. intermediate lesions, with smooth muscle cell migration and proliferation in the inflammation area. In an advanced, complicated lesion, the fibrous cap overlies the lipid and necrotic tissue containing core. Such lesions expand in their shoulder region, where leukocytes adhere (Ross, 1999). For the histological identification of different lesion types, American Heart Association has published instructions for the differentiation of each lesion type (Stary et al., 1994; Stary et al., 1995; Stary, 2000). The initial fatty streak and the intermediate lesion types I, II, and III are able to regress to normality. Most clinical events occur when type VI lesions and also type IV and V lesions are present. These lesion types are non-regressive.

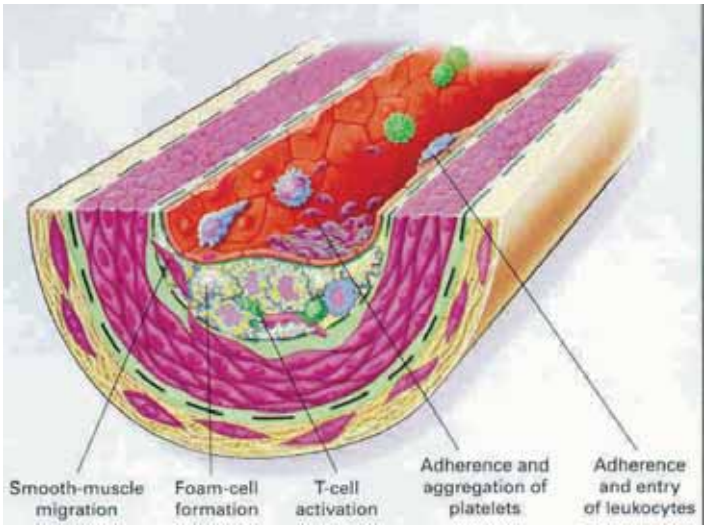


Fig. 4. Fatty streak formation in atherosclerosis development (Ross, 1999). (With permission, Copyright © 2005 Massachusetts Medical Society).

1.5.2 Mouse models for C. pneumoniae *and atherosclerosis*

Mice primarily have a non-atherogenic lipid profile, as most of their cholesterol is carried in high-density lipoprotein (HDL) particles. They are thus quite resistant to atherosclerosis, but disturbances of normal cholesterol metabolism lead to atherosclerotic changes. Atherosclerosis experiments have conventionally been done in wild-type mice by modifying the diet to obtain high fat and high cholesterol concentrations in the circulation. Another approach is genetical alterations of the mouse to have excess cholesterol in the circulation and altered lipid metabolism. ApoE and LDL receptor are important molecules in the clearance of harmful low-density lipoprotein (LDL) cholesterol (Breslow, 1993).

In *C. pneumoniae* mouse models, both genetic and diet-induced approaches have been used. Both extension and progression of lesions have been reported. To obtain chronic infection in mice, repeated exposures with *C. pneumoniae* are used (Grayston et al., 1985; Beatty et al., 1994a). The impact of *C. pneumoniae* infections on atherosclerotic lesions in ApoE-deficient mice has varied, but in other genetically modified mouse strains, studies have shown uniformly that repeated infections have increased either the lesion area or the severity of lesions (Table 3). Even advanced lesions develop in these genetically modified mouse strains.

Table 3. Mouse models for C. pneumoniae *(as a sole pathogen) and atherosclerosis using genetically modified mouse strains.*

Deficiency	Infection		Diet		Impact	Reference
	No	Age*	Age**	Type	on lesions	
ApoE	3	8	-	chow	Increase in area	(Moazed et al., 1999)
ApoE	1-3	na	-	chow	Endoth. dysfunction	(Liuba et al., 2000)
ApoE	3, 4	8	-	chow	No effect	(Aalto-Setälä et al.,
	3, 4	8	na	high fat	No effect	2001)
ApoE	2	6	-	chow	Increase in area***	(Burnett et al., 2001)
ApoE	1, 2	6-8	-	chow	No effect	(Caligiuri et al., 2001)
ApoE	2	10	-	chow	No effect	(Rothstein et al.,
	2	12	-	chow	Increase in area	2001)
ApoE	3	7	-	chow	No effect	(Liuba et al., 2003a)
ApoE3****	2	9	5	high fat	Increase in severity	(Ezzahiri et al., 2002)
LDLR	9	4-5	-	chow	No effect	(Hu et al., 1999)
	9	4-5	na	2% chol.	Increase in area and severity	
LDLR	12	4-6	na	2% chol.	Increase in area	(Liu et al., 2000)
LDLR	4-6	4-6	2-4	2% chol	Increase in area	(Sharma et al., 2004)
ApoE+LDLR	2, 6	16	-	chow	Reduce fibrous cap	(Ezzahiri et al., 2003)
eNOS	3	8	8	high fat	No effect	(Chesebro et al.,
iNOS	3	8	8	high fat	Increase in area	2003)

* age of the mouse at the first *C. pneumoniae* challenge, in weeks

** if a modified diet was used, the age of the mouse when the diet was initiated, in weeks

*** in males only

**** transgenic mouse strain, which has ApoE3-Leiden insertion

Abbreviations used in this table: ApoE=Apolipoprotein E; LDLR=LDL receptor; eNOS=endothelial nitric oxide synthase; iNOS=inducible nitric oxide synthase; na=not assessed; chow=maintenance regular diet; chol=cholesterol.

Among several wild-type strains, the C57BL/6J strain has been shown to be most prone to lesion development when on a high-fat diet (Paigen et al., 1985), although only initial type I and fatty streak type II lesions are seen. In *C. pneumoniae* models, the high-fat diet must be initiated either before the first *C. pneumoniae* inoculation or simultaneously to it to obtain the atherogenic effect of *C. pneumoniae* (Table 4). If a wild-type C57BL/6J mouse is repeatedly infected with *C. pneumoniae* and fed a non-modified diet, only inflammatory changes in the aortic wall are induced.

Table 4. Mouse models for C. pneumoniae *(as a sole pathogen) and atherosclerosis using wild-type mouse strains.*

Mouse	Infection		Diet		Impact	Reference
strain	No	Age*	Age**	Type	on lesions***	
C57BL/6J	1, 3	8	-	chow	Inflammation	(Blessing et al., 2000)
C57BL/6J	3	8	-	chow	No effect	(Blessing et al., 2001)
	3	8	8	high fat	Increase in area	
C57BL/6J	1	6-8	-	chow	No effect	(Caligiuri et al., 2001)
C57BL/6J	3	8	13, 15	high fat	No effect	(Blessing et al., 2002a)
C57BL/6J	3	8	8	high fat	Increase in area	(Chesebro et al., 2003)
C57BL/6J	3	8	6	0.2% chol.	Increase in area	(Erkkilä et al., 2004)
BALB/c***	1-3	6-8	-	chow	Inflammation	(Burian et al., 2001a)

* age of the mouse at the first *C. pneumoniae* challenge, in weeks

** if a modified diet was used, the age of the mouse when the diet was initiated, in weeks

*** the mice were γ-irradiated.

Abbreviations used in this table: chow=maintenance regular diet; chol=cholesterol.

1.5.3 Role of C. pneumoniae *in lesion development*

C. pneumoniae infection may contribute to lesion development in several stages (reviewed in (Mahony and Coombes, 2001)). Being a respiratory pathogen, *C. pneumoniae* may be directly shed into the bloodstream from the lungs or carried within infected monocytes/macrophages through bronchus-associated lymphatic tissue and lymphatics into the circulation (Yang et al., 1995; Moazed et al., 1998; Gieffers et al., 2004b). *C. pneumoniae*–infected monocytes adhere through activation of their integrin adhesion receptor system to noninfected arterial endothelial cells (Kalayoglu et al., 2001; May et al., 2003) and transfer *C. pneumoniae* to endothelial cells (Lin et al., 2000) and further to smooth muscle cells beneath the endothelial cell layer (Rupp et al., 2005), in which *C. pneumoniae* is able to multiply (Kaukoranta-Tolvanen et al., 1994; Godzik et al., 1995; Gaydos et al., 1996). In endothelial cells, *C. pneumoniae* infection induces inflammation (Kaukoranta-Tolvanen et al., 1996; Molestina et al., 1998; Krüll et al., 1999; Molestina et al., 1999; Coombes and Mahony, 2001). Cytosolic Nod proteins participate in the recognition and activation of inflammation due to intracellular *C. pneumoniae* (Opitz et al., 2005). TLR2-dependent activation in endothelial cells occurred only by extracellular *C. pneumoniae* (Opitz et al., 2005). Also, TLR4 has been reported to participate in the recognition of *C. pneumoniae* (Prebeck et al., 2001). cHsp60 and LPS may provide a source for a continuous inflammatory stimulus. Chlamydial LPS gives only a weak inflammatory stimulus, which probably serves to allow persistence. Chlamydial LPS-containing immune complexes are present in acute myocardial infarction patients (Leinonen et al., 1990). cHsp60 can be found in human atheroma, and it induces TNF-α expression (Kol et al., 1998) and activates vascular adhesion molecules as well as the production of proinflammatory IL-6 in vascular cells: endothelial cells, smooth muscle cells, and macro-

phages (Kol et al., 1999). cHsp60-responding T cells are found in the lesion (Curry et al., 2000; Mosorin et al., 2000).

Along with inflammatory activation, *C. pneumoniae* is able to induce the formation of foam cells, which is the initial step in lipid lesion development (Kalayoglu and Byrne, 1998). *Vice versa*, macrophage foam cells can be infected with *C. pneumoniae* (Blessing et al., 2002b). In addition, *C. pneumoniae* and cHsp60, but not LPS, alone were able to induce cellular oxidation of LDL in the supernatant (Kalayoglu et al., 1999).

Smooth muscle cell proliferation is an important step in lesion progression and fibrous cap formation, and *C. pneumoniae* and cHsp60 are able to trigger it (Sasu et al., 2001; Hirono et al., 2003). Although these steps lead to narrowing of the artery, they simultaneously add stability to the atherosclerotic lesion. However, *C. pneumoniae* infection as well as cHsp60 and human Hsp60 (hHsp60) may also induce events that lead to unstability and fibrous cap degradation, by expression and secretion of matrix metalloproteinase, which contributes to tissue damage by proteolytic activity (Kol et al., 1998; Vehmaan-Kreula et al., 2001).

1.5.4 Hsp60 autoimmunity

1.5.4.1 Hsp60 autoimmunity in atherogenesis

The protective stress response may ultimately lead to the development of autoimmune response against the conserved epitopes of self-stress proteins. It may develop as a consequence of repeated microbial exposure and is suggested to be beneficial for the removal of defective host cells, the maintenance of a general level of protection, the first-line defence in infection, and the elimination of stressed cells infected by intracellular bacteria (reviewed by (Young and Elliott, 1989)). Autoimmunity to Hsps may, however, contribute to the pathogenesis of several chronic inflammatory diseases (Zügel and Kaufmann, 1999). Indeed, Hsp60 autoimmunity has been suggested to participate in atherogenesis (Wick et al., 1995). In humans, mycobacterial Hsp65 (mbHsp65) antibodies, which cross-react with hHsp60, were increased in patients with carotid atherosclerosis (Xu et al., 1993b; Xu et al., 1993c). Soluble Hsp60 levels in serum also strongly correlate with atherosclerosis as well as with markers of inflammation and infection (Xu et al., 2000). Parenteral mbHsp65 immunization of normocholesterolemic rabbits leads to atherosclerotic lesion development (Xu et al., 1992), and in combination with a cholesterol-rich diet, advanced lesions develop (Xu et al., 1996). MbHsp65-reactive antibodies and T cells are found in the lesions of mbHsp65-immunized animals (Xu et al., 1993a). Interestingly, mucosal administration of mbHsp65 in high-cholesterol/fat-fed LDL receptor-deficient mice decreases atherosclerotic plaques (Harats et al., 2002; Maron et al., 2002).

1.5.4.2 Chlamydial infection and Hsp60 autoimmunity in atherogenesis

In cHsp60, B-cell and T-cell epitopes conserved and cross-reactive with hHsp60 have been detected (Yi et al., 1993; Witkin et al., 1994). Self-Hsp60-reactive antibodies and T cells are induced in mice simultaneously immunized with cHsp60 and mouse Hsp60 (mHsp60), but not if either agent is given alone (Yi et al., 1997). In humans, cross-reactive Hsp60 antibodies exist (Domeika et al., 1998). In atherosclerosis, mbHsp65 immunity correlates with *C. pneumoniae* and *Helicobacter pylori* infections (Mayr et al., 2000), and high levels of immunoglobulin G (IgG) antibodies to both cHsp60 and hHsp60 are independent and combined risk factors for atherosclerosis (Burian et al., 2001b; Mahdi et al., 2002). In addition, cHsp60 IgG antibodies correlate with the detection of *C. pneumoniae* in human atheroma (Fong et al., 2002). For coronary risk, elevated levels of hHsp60 IgA, *C. pneumoniae* IgA and CRP are powerful predictors when present together, and especially when persistently elevated (Huittinen et al., 2002; Huittinen et al., 2003).

The mechanisms in atherogenic Hsp60 autoimmunity are proposed to act through reactivity with Hsp60 expressed on the surface of stressed endothelial cells and endothelial cytotoxicity (Xu et al., 1994; Schett et al., 1995) Hsp60 expression is increased in endothelial cells in atherosclerotic lesions in humans and in rabbits, but not in disease-free rabbit vessels (Kleindienst et al., 1993; Xu et al., 1993a). In experimental animals, the expression of Hsps in aortic endothelial cells is enhanced in stress condition, such as cholesterol-rich diet (Xu et al., 1993a), hypercholesterolemia (expression correlated with the development of lesions) (Kanwar et al., 2001), exposure to LPS (Seitz et al., 1996), fluctuation in blood flow (Hochleitner et al., 2000), or *in vitro* in monocytic cells by oxidized LDL (Frostegard et al., 1996). *C. pneumoniae* infection in endothelial cells also uppregulates Hsp60 expression (Shi and Tokunaga, 2004). Hsp65/60 antibodies that cross-react with hHsp60 and cHsp60, as well as *E. coli* GroEL, are able to mediate endothelial cytotoxicity (Mayr et al., 1999).

1.5.5 Statins and their antimicrobial effects

Statins are lipid-lowering drugs that are widely used to prevent cardiovascular diseases (reviewed in (Maron et al., 2000)). They competitively inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the mevalonate pathway of isoprenoid biosynthesis, which is also the rate-limiting enzyme in the cholesterol biosynthesis pathway. This inhibition ultimately leads to decreased LDL cholesterol levels in serum (Endo et al., 1977; Corsini et al., 1995). In addition to their lipid-lowering effect on circulating and lesional lipids, statins have other effects also considered protective against atherosclerosis and its complications, e.g. immunomodulation, reduction of cellular activition and proliferation, and improvement of endothelial function and vasomotion (reviewed in (Vaughan et al., 1996)). It has been shown that pravastatin also decreases coronary events in patients with average cholesterol levels (Sacks et al., 1996) and with slightly elevated CRP levels, and further, that they decrease elevated CRP levels (Ridker et al., 1999; Strandberg et al., 1999). Several immunomodulatory effects have been re-

ported (Kwak et al., 2000; Loike et al., 2004; Schonbeck and Libby, 2004), and both innate and acquired types of immunity are affected. *In vivo*, the influx of different leukocytes, PMN leukocytes, eosinophils, macrophages, and lymphocytes due to inflammatory stimuli is inhibited, (Diomede et al., 2001; Sparrow et al., 2001; McKay et al., 2004; Williams et al., 2004; Yeh and Huang, 2004). Different statins may, however, have different immunomodulatory effects (Kiener et al., 2001).

Through inhibition of HMG-CoA reductase in the mevalonate pathway, statins also inhibit isoprenoid biosynthesis in eucaryotes and also in some bacteria (Hedl et al., 2002). *C. pneumoniae* does not have the HMG-CoA reductase enzyme, but *in vitro* studies have shown that cerivastatin tends to reduce infection rates in macrophages, and the secretion of monocyte chemoattractant protein-1 (MCP-1) and IL-8 decreased both in macrophages and endothelial cells (Kothe et al., 2000). Dechend *et al*. have shown that cerivastatin reduces *C. pneumoniae* infection of smooth muscle cells via infected human macrophages and blocks cell membrane-associated RhoA and Rac1 through inhibition of prenylation, reactive oxygen production, and RANTES and MCP-1 (Dechend et al., 2003). Statins also reduce the intracellular growth of *Salmonella enterica* serovar Typhimurium *in vitro* and *in vivo* in mouse (Catron et al., 2004) and inhibit *in vitro* the growth of two viruses, cytomegalovirus (Potena et al., 2004) and HIV-1. Inhibition of HIV-1 also occurred *in vivo* in infected patients (del Real et al., 2004). In a sepsis mouse model (gram-negative rods, gram-positive cocci), simvastatin treatment improved survival and reversed increased monocyte adhesion to endothelium (Merx et al., 2004), and in a LPS-induced sepsis model, cerivastatin improved survival and reduced the levels of inflammatory mediators (Ando et al., 2000). In humans, too, statin treatment has been shown to reduce mortality in bacteremic patients (Liappis et al., 2001) and to reduce the rate of severe sepsis (Almog et al., 2004).

2 Aims of the study

The present project was carried out using mouse models for *C. pneumoniae* infection. The specific aims were to analyze:

1. Technical aspects of the mouse model, i.e. the impact of the anesthetic agent on inoculating the mice and the presence of host cell remnants in the inoculum

2. Pulmonary infection and inflammation caused by different *C. pneumoniae* isolates administered via different inoculation routes and during cholesterol feeding

3. Development of cHsp60 and mHsp60 antibody responses and development of atherosclerotic lesions

4. Effects of simvastatin treatment on immune and inflammatory responses and *C. pneumoniae* growth

3 Materials and methods

3.1 Mouse model

3.1.1 Mice and diets

The Animal Care and Use Committee of National Public Health Institute, Helsinki, approved all procedures involving experimental animals in this project. The following mouse strains were used in the experiments: female inbred C57BL/6JBom mice (M&B A/S, Ry, Denmark) (I, III) and female outbred NIH/S mice (National Public Health Institute, Kuopio, Finland) (I, II, IV). The mice were specifically pathogen-free, and they were maintained under pathogen-free conditions and handled using Biosafety level-II precautions. The temperature, relative humidity, number of air changes, and photoperiod were automatically controlled in the animal facilities.

The mice were allowed an autoclaved or irradiated regular chow diet (Altromin 1324, Chr. Petersen A/S, Ringsted, Denmark) and water *ad libitum*. In study III, a group of mice were fed an autoclaved regular chow diet with 0.2% cholesterol supplement. In study IV, a group of mice were fed a high-fat diet consisting of 21% total fat, 0.2% cholesterol, and 19.5% casein (Harlan Teklad, Horst, The Netherlands).

3.1.2 C. pneumoniae *challenge*

The mice were given an infectious challenge i.n. under light carbon dioxide (CO_2) or methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL, USA) anesthesia. Control mice were given similarly sucrose-phosphate-glutamate (SPG) solution. Two different *C. pneumoniae* isolates, which are both throat swab isolates, Kajaani 6 (K6) (I, II) or Kajaani 7 (K7) (I, III, IV), from an epidemic in Finnish military conscripts (Ekman et al., 1993) were used to

challenge the mice. Both isolates have been propagated in HL (a human epithelial cell line) cells and stored in SPG solution at -70°C (National Public Health Institute, strain collection). The infectious challenge was a crude chlamydial preparation consisting of *C. pneumoniae* particles, mainly EBs, and HL host cell remnants. The infectious dose per mouse, which was quantified directly after the challenge, varied between $1.8x10^5$ and $2.0x10^6$ inclusion-forming units (IFU) in different experiments.

In one experiment, a cellular preparation consisting of HL host cell remnants was given to a control group of NIH/S mice and also before or after the *C. pneumoniae* challenge (II). In study III, C57BL/6J mice were fed either a regular chow diet or a regular chow diet with 0.2% cholesterol supplementation and repeatedly infected. The 1st reinfection was given 7 weeks after the primary infection, and the 2nd reinfection was given 11 weeks after the primary infection, i.e. 4 weeks after the 1st reinfection (III). A separate experiment was also performed where a group of C57BL/6J mice were infected i.n. and another group intragastrically (i.g.). The i.g. inoculation was given under Hypnorm sedation.

3.1.3 Other treatments (IV)

In study IV, a group of NIH/S mice on a regular chow diet and a group on a high-fat diet were treated with simvastatin. Simvastatin (L-644, a gift from Merck) was dissolved in 1 % dimethylsulfoxide (1 % DMSO), and the selected daily dose (0.5 mg/kg) was given as i.p. injections (100 µl) for 24 consecutive days. Control groups of NIH/S mice were treated with the same volume of vehicle.

3.2 Detection of *C. pneumoniae*

3.2.1 Chlamydial culture (I, II, III, IV)

For the detection of infective chlamydia in different tissues of the mice, the tissue samples were mechanically homogenized (Stomacher blender) in SPG, and the supernatant of the tissue homogenate was inoculated in several volumes into either HL or Vero cells (Green monkey kidney cell line) grown in 24-well plates. After pipetting the samples into the wells, the plates were centrifuged at 550 x g for 1 h, and the the media were replaced with media containing 0.5 µg/ml cycloheximide and incubated for 72 hours. Cells were fixed with methanol and stained with fluorescein isothiocyanate-conjugated chlamydia LPS-specific antibodies (Pathfinder Chlamydia Confirmation System, Kallestad Diagnostics, Chaska, MN).

3.2.2 Detection of C. pneumoniae *DNA by PCR (II, III, IV)*

For the detection of *C. pneumoniae* DNA from samples of mouse tissue, DNA was extracted using the QIAamp Tissue Kit (Qiagen GmbH) by following the manufacturer's instructions. One to 10 µl of DNA extract was used for the PCR reaction. Three different PCR protocols were used in this project. In the first PCR protocol (II), the PCR reaction was performed using the *C. pneumoniae* 16S rRNA gene-specific primers CpnA and CpnB, which result in a 463 bp product (Gaydos et al., 1992). The reaction was done in a 25 µl mixture with AmpliTaq GOLD polymerase (PE Biosystems), and the cycle number was 40. The PCR products were visualized in a 2% agarose gel electrophoresis. The second PCR protocol (III), hot-start PCR, was performed using *C. pneumoniae omp1* -specific biotinylated primers (Institute of Biotechnology, Helsinki, Finland) with AmpliTaq polymerase (Promega) and AmpliWax Gem 50 bed (PE Biosystems) in a total reaction volume of 50 µl. The cycle number was 50. The presence of a specific PCR product was confirmed by measuring time-resolved fluorescence in liquid hybridization with Eu-labeled SJ1 probes (Institute of Biotechnology) in streptavidin-coated microplates (Wallac). The third (IV) and fourth (i.g. infection experiment) PCR protocols were real-time quantitative protocols for Light Cycler machine (Roche), using *pmp4* (Mygind et al., 2001) and 16S rRNA (Gaydos et al., 1992) -specific primers and a hybridization probe in a total reaction volume of 25 µl (Reischl et al., 2003). The number of cycles was 50.

3.3 Inflammation

3.3.1 Pulmonary inflammation (II, III, IV)

Histopathological analysis was performed on lung samples. The tissue samples were fixed using phosphate-buffered formaldehyde and embedded in paraffin. Sections of 4 µm in thickness were cut from the paraffin blocks using a microtome, and the sections were stained with hematoxylin and eosin. The degree of pulmonary inflammation was assessed from the mononuclear and polymorphonuclear leukocytes present in the stained sections. Grading depended on the number of cells and the area affected. In the mild forms, the inflammatory cell infiltrates were limited to the focal areas or occurred in small scattered foci, while in the severe cases, large tissue areas were affected.

3.3.2 Circulating inflammatory markers (IV)

Serum IL-10 levels were measured using the Quantikine® M mouse IL-10 immunoassay kit according to the manufacturer's instructions (i.g. experiment, R&D Systems). Serum amyloid A (SAA) concentrations were measured using a commercial EIA method (IV, BioSource International, Camarillo, CA, USA), and serum LBP concentrations were

measured from pooled (n=6) mouse sera (HyCult Biotechnology b.v., Uden, The Netherlands).

3.4 *C. pneumoniae*-specific and autoimmune responses (II, III, IV)

The sera of mice were tested for *C. pneumoniae*-specific IgG antibodies using enzyme immunoassay (EIA). Commercial plates coated with whole *C. pneumoniae* EBs were used (AniLabsystems, Helsinki, Finland) in accordance with the manufacturer's instructions. Mouse sera were tested in 1:100 dilution, and for the detection of bound IgG antibodies, alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:3000 with PBS containing 10% fetal bovine serum was used.

From the lungs, using a supernatant of lung tissue homogenate, *C. pneumoniae*-specific IgA antibodies were measured using EIA and commercial plates coated with whole *C. pneumoniae* EBs. The supernatant was diluted 1:5, and for the detection of IgA antibodies, alkaline phosphatase-conjugated rabbit anti-mouse IgA (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:1000 with PBS containing 10% fetal bovine serum was used.

C. pneumoniae Hsp60-specific IgG antibodies were measured. In EIA, recombinant *C. pneumoniae* Hsp60 protein produced in *Bacillus subtilis* was used as an antigen ((Airaksinen et al., 2003), kindly provided by professor Matti Sarvas, National Public Health Institute, Helsinki, Finland). The serum samples were diluted 1:1000 and bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse immunoglobulin diluted in PBS-Tween (Sigma).

Serum IgG autoantibodies against mHsp60 were measured by EIA using recombinant mHsp60 protein as an antigen (StressGen Biotechnologies Corp, Canada). The serum samples were diluted 1:10, and bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse immunoglobulin (Sigma).

3.5 Quantification of atherosclerotic lesions and serum lipids (III, IV)

A method of lesion quantification described by Paigen et al and computer-assisted morphometric measurement were used to quantitate lipid lesions (Paigen et al., 1987). The upper part of the heart was fixed in 10% phosphate-buffered formalin and embedded sequentially in 5%, 10%, and 25% gelatin. The sections were cut in a plane parallel to the atria. Every second of the consecutive 12-14 μm frozen sections was stained with Oil-Red-O lipid stain, and lipids were counterstained with hematoxylin and Light green. True-color digital images were captured with a ColorView 12 video camera (resolution 1300 x 1300) attached to an Olympus BX-50-F3 light microscope with 20x or 40x objectives. Every third stained section was analyzed for Oil-Red-O stainings using the analySIS software (Soft Imaging System, GmbH). The number of sections (n=4) taken for statistical analyses was chosen according to the lesions that extended most widely along the ascending aorta. The data are expressed as a geometric mean of the Oil-Red-O-stained lesion areas with a 95 % confidence interval.

Serum total cholesterol and triglycerides were measured with fully enzymatic methods (Roche Diagnostics, Basel, Switzerland and Wako Chemicals GmbH, Nuess, Germany).

3.6 Statistics

To determine the statistical differences, non-parametric Mann-Whitney U-test, $\chi 2$ test, or 2-sided Fisher's Exact Test was used. *P*-values of <0.05 were considered statistically significant.

4 Results

4.1 Technical considerations of *C. pneumoniae* mouse model

4.1.1 Effect of anesthetic agent (I)

Originally, in this mouse model, the mice have been immobilized with CO_2 for effective delivery of intranasal inoculation. In the present study, methoxyflurane, which is used in short- and long-term surgical anesthesia (Danneman and Mandrell, 1997), was compared to CO_2. C57BL/6J mice were inoculated i.n. with K6 isolate. In the C57BL/6J mice inoculated i.n. under methoxyflurane anesthesia, chlamydial culture findings in the lungs showed more consistent infection at the days 6 and 12 postinfection (p.i.) than in the mice that had been infected under CO_2 (I, Table 1).

4.1.2 Effect of host cell debris in challenge inoculum (II)

For inoculation of mice with infective *C. pneumoniae*, a crude preparation, which also contained host cell components, was used instead of highly purified EB preparations. Possible inflammatory or immune responses against host cell components, such as Hsps, were recorded. The results show that only some mild lymphocyte infiltrations were seen in the lungs, and no serum IgG response against *C. pneumoniae* or mHsp60s developed (II, Table 1).

When the mock-inoculated mice were infected 30 days later with K6, the course of infection resembled primary infection, and the chlamydial titres in the lungs were lower (day 3 p.i., median 0.7 IFU/sample $\log_{10}$) compared to the lungs of mice which had received two K6 infections (day 3 p.i., median 1.5 IFU/sample $\log_{10}$) (II, Table 1). PCR positivity correlated with the culture findings in each group. The inflammatory reaction in

the lungs, however, was milder compared to the primary infection without mock pretreatment and milder than in normal *C. pneumoniae* reinfection, where the cellular inflammatory response in the lungs is fast and strong. (II, Table 1, Fig. 1). In each study group, the inflammation began to subside at day 28-30 at the latest. *C. pneumoniae* Hsp60 IgG levels rose, as did IgG antibodies against the whole EBs. No mHsp60 IgG antibodies were detected.

A group of mice were primarily infected with *C. pneumoniae*, and the culture-negative mice were inoculated 30 days later with a host cell preparation. After the host cell inoculum, reactivation of infection was detected by culture (II, Table 1). *C. pneumoniae* PCR from lung tissue was also positive, and the lung inflammation was enhanced compared to the primary mock inoculation or the mock/mock rechallenge, but was milder than after a *C. pneumoniae* reinfection (II, Table 1). In addition to showing IgG antibodies against whole *C. pneumoniae* EBs and *C. pneumoniae* Hsp60, some of these mice also developed elevated mHsp60 IgG levels (II, Fig. 2).

4.2 Pulmonary infection and inflammation

4.2.1 Comparison of the rates of growth of two different C. pneumoniae *Kajaani isolates (I)*

Both of the Kajaani isolates used in the present studies, K6 and K7, are consecutive throat swab isolates that have been propagated in HL cells. The K6 isolate has been used in mouse and K7 in rabbit studies. The two isolates were now compared in parallel, and slightly different infection courses were demonstrated by chlamydial cultures from the lungs of NIH/S mice after i.n. infection: inoculation with the K7 isolate led to higher chlamydial titers at the early stage than inoculation with the K6 isolate, while opposite results were obtained at later time points (I, Table 2).

4.2.2 Development of pulmonary infection and inflammatory cell infiltrations after different challenging routes

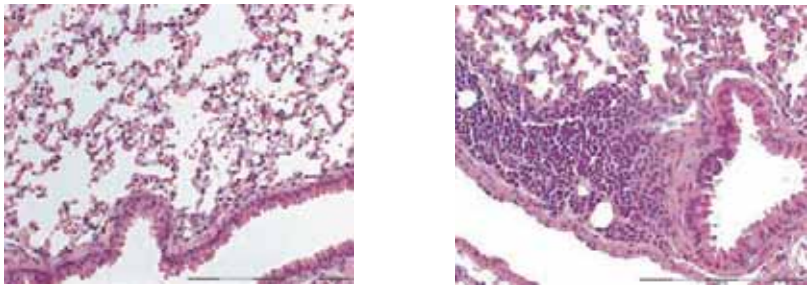
The C57BL/6J mice were infected either i.n. or i.g. After i.n. infection, chlamydia could be cultured in large amounts from the lungs, and PCR positivity was also high. The inflammation in the lungs, which was seen as patchy mononuclear cell infiltrations around the bronchi and veins, was severe and continued till the end of the follow-up. On the contrary, after i.g. infection, infective chlamydiae were not cultured from any of the tissues, including lungs, liver, and rectal swab, but PCR was positive in a few lung and liver samples (Table 5). In addition, mild lymphocyte infiltrations were detected in the lungs (bronchointerstitial pneumonia) at day 9 p.i., and they increased steadily up to day 34 p.i.

(Fig. 5). In addition, in both groups of mice, IL-10 levels in serum were slightly elevated at the early time points, and in i.g.-infected mice, a second peak occurred after the lymphocyte infiltrations at day 14 p.i.. *C. pneumoniae* IgG antibodies in serum developed (OD>0.10) after i.n. infection at day 9 p.i., and in i.g.-infected mice at day 24 p.i. In the lungs, *C. pneumoniae* IgA antibodies were present after i.n. infection, but not after i.g. infection.

Table 5. Incidence rates (pos./studied) of C. pneumoniae *PCR-positive lung and liver samples of. primarily i.n. and i.g infected C57BL/6JBom mice at different time points.*

Days postinfection	Lung		Liver	
	i.n.	i.g.	i.n.	i.g.
3	6/7	0/7	7/7	2/7
6	5/7	1/7	5/7	0/7
9	5/7	0/7	4/7	0/7
14	3/7	1/7	3/7	1/7
24	6/7	3/7	2/7	0/7
34	5/7	1/3	0/7	1/7

Abbreviations used in this table: i.n.=intranasal; i.g.=intragastric.



A B

Fig. 5. Hematoxylin and eosin stained lung section from A) a control mouse showing normal histopathology of C57BL/6J mouse lung, and B) from an i.g. infected mouse showing mononuclear cell/lymphocyte infiltrates.

4.2.3 Effect of cholesterol feeding on chronic C. pneumoniae *pulmonary infection (III)*

C57BL/6J mice, which were fed either a regular chow diet or a regular chow diet with 0.2% cholesterol supplementation, were repeatedly infected with K7. *C. pneumoniae* DNA could be detected by PCR at each time point, and the rate varied between the different diet groups (III, Fig. 6). After the first reinfection, the rate tended to be higher in the mice on a cholesterol-enriched diet than in those fed a regular diet, but after the second reinfection, DNA positivity was slightly lower in the mice on a cholesterol-enriched diet compared to those fed a regular diet. In the other tissues studied, i.e. hearts, aortic arches,

and abdominal aortas, *C. pneumoniae* DNA was detected intermittently, and in all, the DNA positivity was slightly higher in the group of mice fed a cholesterol-enriched diet than in the regular diet group.

In the lungs of repeatedly infected C57BL/6J mice, *C. pneumoniae* induced typical inflammatory changes, i.e. infiltrates of mononuclear cells mainly around the bronchi and veins (III, Fig. 4). These inflammatory cell infiltrates persisted up to the last time point studied, i.e. 12 weeks after the second reinfection. The cholesterol-enriched diet slightly exaggerated the inflammation at the time points after the first reinfection and up to 8 weeks after the second reinfection (III, Fig. 5).

4.3 Development of Hsp60 responses and atherosclerotic lesions

4.3.1 Chlamydial and mHsp60 responses (II, III)

When NIH/S mice were primary infected with K6 and reinfected 30 days later, the serum IgG antibody levels against *C. pneumoniae* Hsp60 started to rise at day 10 p.i. The levels further rose after the reinfection. The *C. pneumoniae* Hsp60 IgG levels correlated with the respective IgG antibody levels against whole *C. pneumoniae* EBs (r=0.9)(Fig. 6).

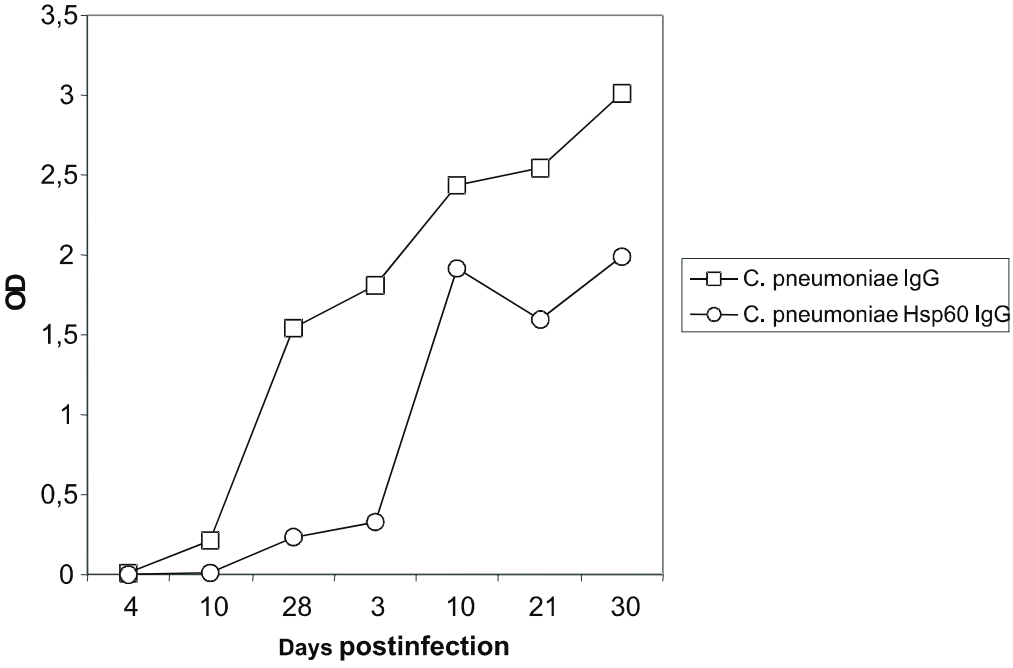


Fig. 6. *C. pneumoniae* IgG and *C. pneumoniae* Hsp60 IgG levels. NIH/S mice were primary infected and reinfected 30 days later. Different serum dilutions were used (for details, see chapter 3.4).

In the reinfected NIH/S mice, highly elevated levels (OD 405nm > 0.5) of mHsp60 IgG antibodies were also present in some individuals (II, Fig. 2). Most of the mice with elevated levels also had high *C. pneumoniae* Hsp60 antibodies present in serum. However, not all mice with high *C. pneumoniae* Hsp60 IgG levels had mHsp60 IgG antibodies in their serum. mHsp60 IgG was also found in the repeatedly infected C57BL/6J mice that were fed a regular chow diet or a regular chow diet with 0.2% cholesterol supplementation (III, Fig. 2). These IgG autoantibody levels were significantly increased in reinfected mice, and a faster increase was detected in the mice fed a cholesterol-enriched diet compared to those fed a regular diet. The cholesterol-enriched diet even alone increased the levels transiently.

4.3.2 Aortic lesions and serum lipids (III)

Foam cell lesions of two different types were detected in the aortic valve cross-sections from repeatedly infected (K7) C57BL/6J mice. One type was present exclusively in the media layers in the arterial walls between valve cusps and was not enhanced by *C. pneumoniae*. Another lesion type predominated in the subendothelium at valve cusps and resembled individual and aggregated foam cells in the intima layer (III, Fig. 3). These foam cell lesions were significantly increased by the repeated *C. pneumoniae* infections compared to SPG inoculations of the mice on a cholesterol-enriched diet; 116 μm^2 (95% CI 32-416 μm^2) versus 7 μm^2 (95% CI 1-62 μm^2), P=0.022, respectively. Only occasional foam cells were detected in the mice on a regular diet independently of the *C. pneumoniae* infection, 2 μm^2 (95% CI 1-5 μm^2) in both groups.

The cholesterol-enriched diet with 0.2% cholesterol supplementation did not affect the serum lipid levels, triglycerides, or total cholesterol. Nor were there differences in these circulatory lipid levels due to *C. pneumoniae* infections.

4.4 Effect of simvastatin

4.4.1 Effect of simvastatin treatment on the growth of C. pneumoniae *in mouse lungs (IV)*

In the simvastatin-treated NIH/S mice, infective chlamydiae were detected in decreased amounts compared to the vehicle-treated mice. These reductions (55-82%) were seen at the early stages of chlamydial infection (days 3 and 6 p.i., respectively) both in the mice fed a regular diet and in those fed with a high-fat diet (Fig. 7 and IV, Fig 2). At later time points, no marked differences in the counts of viable chlamydiae due to simvastatin treatment could be detected. By quantitative PCR, the amount of *C. pneumoniae* DNA

also decreased at same time points and to the same degree as the amount of infective chlamydiae (Fig. 7).

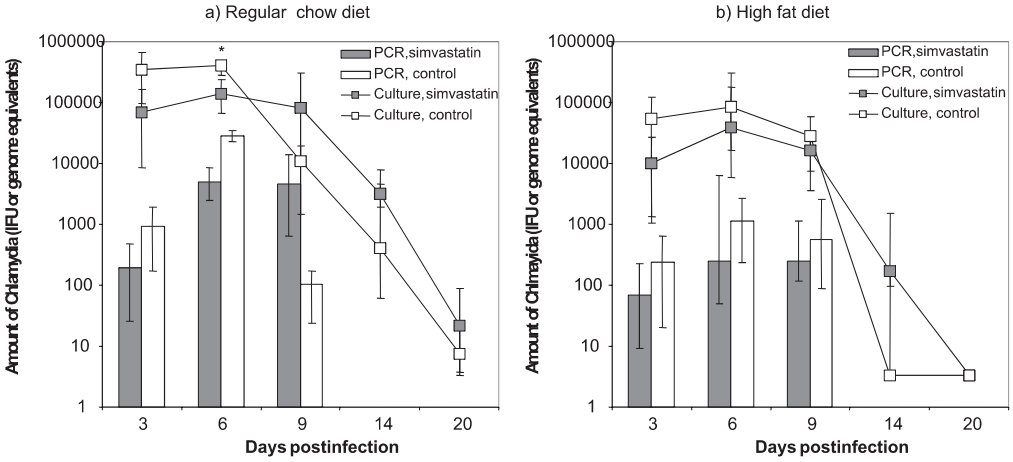


Fig. 7. Chlamydia culture (IFU) and *C. pneumoniae* PCR (genome equivalent) results from the lung tissue of mice fed a) a regular chow diet, or b) a high-fat diet. The results are shown as median±interquartile range (IQR, 25th and 75th percentile). Culture results were calculated for the whole right lung and PCR results for 8 µl of DNA extract. * P=0.026.

Simvastatin treatment of mice did not lower the serum lipid levels of either triglycerides or total cholesterol. The high-fat diet caused a rise of the serum lipid levels. (IV, Table 1).

4.4.2 Immunomodulatory effect of simvastatin treatment (IV)

In the sera of infected mice, a moderate increase in the concentrations of acute phase proteins was seen. Both SAA and LBP levels started to rise at day 3 p.i. and were highest at day 6 p.i.. The increase of SAA was over 200-fold and that of LBP about 10-fold. Toward the days 14-20 p.i., the levels returned to the baseline. Simvastatin treatment did not affect the levels significantly.

Simvastatin treatment did not affect the *C. pneumoniae*-specific IgG antibody response, either. There was, however, a slight increase in the levels in the high-fat mouse group, but the background levels in the sera of the mice on a high-fat diet were already significantly higher at the beginning of the experiment compared to those in the sera of the mice on a regular diet, and the finding was thus considered an artefact (increased turbidity of lipemic sera). In the lungs, the effect of simvastatin treatment on the course of infection was seen as an increase of mononuclear cell infiltrates (IV, Fig. 2). In the mice fed a regular diet, at each time point, there was a slight increase in these infiltrates due to the simvastatin treatment compared to the vehicle-treated mice, and an increase in the infiltrates due to the simvastatin treatment was also seen in the mice on a high-fat diet.

5 Discussion

5.1 Technical considerations of the *C. pneumoniae* mouse model

In *C. pneumoniae* mouse models, CO_2, ketamine+xylazine, and methoxyflurane have mostly been used as anesthetizing agents. Two different anesthetics, CO_2 and methoxyflurane, were compared here in inoculating mice i.n. with *C. pneumoniae*. The use of methoxyflurane led to a more consistent distribution of bacteria in the respiratory tract in individual mice and to higher chlamydial titers. Interestingly, in a pneumococcal pneumonia mouse model, too, the use of inhaled anesthetics, methoxyflurane or halothane, in i.n. infection led to higher bacterial counts in the lungs compared to anesthesia with an i.p. injection of pentobarbital (Rubins and Charboneau, 2000). That study further showed differences in the levels of pro-inflammatory cytokines, which were not assessed in the present study. The depth of anesthesia was easily controlled with methoxyflurane, and the mice breathed normally without gasping, which is frequently seen during CO_2 anesthesia. CO_2-anesthetized mice showed more variation in culture positivity. However, the high antibody levels also seen in culture-negative mice, both CO_2- and methoxyflurane-anesthetized, indicate that they have all become infected with chlamydia (data not shown). Thus, it is possible that, due to incomplete anesthesia with CO_2 in some mice, most of the chlamydial inoculum may have remained in the upper respiratory tract, and too few organisms may have entered the lung to be detected with lung tissue culture.

When chlamydiae are grown in different hosts and tissues, the composition and infectivity of the chlamydial preparations is affected (Kuo et al., 1975; Kordova et al., 1977; Allan and Pearce, 1979; Neuman et al., 1980). As demonstrated in early human vaccination trials with *C. trachomatis*, and later in monkeys (MacDonald et al., 1984; Grayston et al., 1985), vaccination with whole organisms led to a more exaggerated disease during a natural *C. trachomatis* infection compared to nonvaccinated subjects. Although the vaccine preparations used in the above studies may have contained host components, the exaggerated disease after repeated chlamydial infections has been well documented later. The crude chlamydial preparations used in most mouse studies certainly contain more cellular contaminants than vaccine preparations, but based on the results of the present

study, the main factors associated with exaggerated pathology in the present *C. pneumoniae* mouse model are not those included in the host cell components. When mice were i.n. mock-inoculated with cellular components alone, mild inflammation was detected in the mouse lungs. In BALB/c mice, i.n. inoculation with cell components has also been reported to induce only mild histological changes in the lungs (Stack et al., 2000), but no IL-12, IFN-γ, TNF-α, IL-10, or IL-4 production (Geng et al., 2000a). In the present study, IgG antibodies against mHsp60 were not produced after mock inoculation. However, the cellular preparation consisted of HL cell components, and HL is a cell line of human origin. Thus, no mHsp60 antibodies were expected to be produced, although there is high sequence identity between the conserved epitopes of mHsp60 and hHsp60s to allow cross-reaction between these and mammalian Hsp60s. Instead, the mock inoculation may have induced local antibody production against the cellular components, and the reinoculation with the crude chlamydial preparation may have activated the immunological memory mechanisms against cell components, because a partial, although low, protective effect against consecutive *C. pneumoniae* infection was detected. The protection was also seen in histology.

An important finding was the reactivation of culture-negative *C. pneumoniae* infection with a cellular mock preparation. In this mouse model, after i.n. inoculation of the mice with a *C. pneumoniae* challenge dose containing approximately 10^6 IFUs, the lungs of NIH/S mice usually only remain culture-positive for 30 days p.i.. However, it has been shown earlier that a culture-negative persistent infection in the lungs can be reactivated as culture-positive by immunosuppressive cortisone treatment (Malinverni et al., 1995a; Laitinen et al., 1996). A boosting effect of *C. trachomatis* infection on *C. pneumoniae* IgG levels, but not cultures, after two *C. pneumoniae* infections in primates has been reported (Holland et al., 1990). Mock reactivation importantly indicates that a latent infection can be reactivated by environmental stimuli in addition to immunosuppression with cortisone. Intermittent detection of *C. pneumoniae* is frequently seen in animal models with prolonged follow-up times (Moazed et al., 1997). In humans, too, periods of PCR positivity and PCR negativity have been reported (Falck et al., 1996). In addition, *C. pneumoniae* IgA reactivation has been associated with inflammation in the airways in acute asthma (Wark et al., 2002). On the other hand, it is also possible that no reactivation actually took place here, but that the chlamydia had remained viable for 34 days after the primary infection, and the culture positivity merely reflected the prolonged primary infection.

5.2 Aspects of *C. pneumoniae* pulmonary infection and inflammation

Though *C. pneumoniae* strains possess only one serotype, antigenic variation and differential expression of surface proteins occur (Black et al., 1991; Christiansen et al., 1999). Possible microbial polymorphism concerning the virulence of *C. pneumoniae* strains is still not well known. In mouse models, parallel comparison of isolates has shown different infection courses between isolates (Kaukoranta-Tolvanen et al., 1993). In the present study, a difference between two Finnish *C. pneumoniae* isolates, K6 and K7, in the recovery of viable organisms from lung tissue of NIH/S mice was demonstrated. This could be due to the small structural differences between the isolates. On the other hand, K6 and

K7 were isolated from consecutive patients during a *C. pneumoniae* epidemic in Finland, and they have been propagated in HL cells, suggesting that the isolates are closely related, and that some other factors such as the number of passages may be involved. The two isolates have not been sequenced. Thus, the conclusions concerning the possible structural or other genetic differences between the two isolates and the consequences on the course of infection are not proven but inconclusive. In addition, we do not know yet if these two Finnish isolates harbor the already identified atherogenic properties or have as yet unidentified ones. And several hypothetical proteins without known function and homologs in other organisms exist in *C. pneumoniae*, but have not yet been characterized.

C. pneumoniae disseminates into different organs after i.n., i.v., and s.c. inoculation of experimental animals. In primates, *C. pneumoniae* is detectable in the rectum of animals infected into the conjuctiva or nasopharynx (Holland et al., 1990). In the intestinal mucosal biopsies in humans, too, *C. pneumoniae* can be detected with PCR (Chen et al., 2002). The i.g. challenging route for *C. pneumoniae* in animal models has not been reported earlier, but *C. trachomatis* MoPn (*C. muridarum*), a mouse pathogen, is able to cause a persistent gastrointestinal infection/colonization in mouse (Igietseme et al., 2001). When the infectious challenge was given to the mice i.g. here, *C. pneumoniae* DNA could be detected in their liver and lungs. In addition, inflammation (lung histology, IL-10 levels) and a humoral immune response against *C. pneumoniae* (serum IgG) could be demonstrated. Contrary to *C. trachomatis* MoPn, which is able to cause a persistent gastrointestinal infection/colonization in mouse (Igietseme et al., 2001), viable chlamydiae could not be cultured from rectal swabs. It is, indeed, possible that there were too few viable chlamydiae in the gastrointestinal tract to be detected by culture from the rectum. This culture negativity of rectal swabs in the present study indicates that no autoinoculation took place. Development of peribronchial and perivascular mononuclear cellular infiltrates could indicate either the development of pulmonary infection with actively replicating *C. pneumoniae* in the lungs or the recruitment of homing lymphocytes into distinct mucosal sites, e.g. the lungs, as a function of the common mucosal system and without active pulmonary infection (Quiding-Jarbrink et al., 1997).

In C57BL/6J mice, repeated challenges with *C. pneumoniae* K7 led to persistent infection, as demonstrated by PCR and histology. DNA was present in lung tissue with variable PCR positivity rates, suggesting the insidious nature of infection or the difficulty of recovering *C. pneumoniae* DNA uniformly from tissue samples (Chernesky et al., 2002). The mice on a cholesterol-enriched diet had slightly increased PCR positivity in their heart and lung at early time points, but this effect was lost and even reversed at later time points. Previously, atherogenic high-cholesterol diet has been shown to increase the presence of *C. pneumoniae* antigen in the aorta of mice (Hu et al., 1999). The effect of cholesterol feeding on *C. pneumoniae*-induced pulmonary inflammation has not been reported earlier, though other mouse models have shown that hypercholesterolemia impairs innate immunity, suppresses T cell responses, hence increasing infectious mortality (Roselaar and Daugherty, 1998; Ludewig et al., 2001), as well as changes the Th1/Th2 balance during the development of atherosclerotic lesions in ApoE-deficient mice (Zhou et al., 1998). In the present study, the pulmonary inflammation in repeatedly infected mice was slightly intensified by a cholesterol-enriched diet during the first reinfection and in the acute phase (4-8 weeks) of the second reinfection. Thus, even though the mice in the present study were not overtly hypercholesterolemic, the findings suggest that even

a small cholesterol increment in the diet has deleterious effects on the course and immunopathology of *C. pneumoniae* infection.

5.3 Chlamydial infection and induction of immune response against Hsp60s

A clear IgG antibody response against *C. pneumoniae* Hsp60 developed in infected NIH/S mice, and the levels correlated with those against whole EBs. No comparisons with the severity of infection (culture or pulmonary inflammation) could be performed due to the small group sizes. In a *C. trachomatis* monkey model, the persistence of cHsp60 antibodies correlated with chlamydial presence, and further, high levels correlated with more severe pathology (Peeling et al., 1999).

Importantly, autoantibodies against self-Hsp60 also developed in the mice of the present study. Subcutaneous immunization of mice with heat-inactivated *Mycobacterium tuberculosis* or mbHsp65 has been found to induce formation of antibodies reacting with mbHsp65 and to accelerate atherosclerosis (George et al., 1999; Afek et al., 2000; George et al., 2000). Furthermore, the immunization of LDL receptor-deficient mice with ovalbumin (OVA) fed a cholesterol-enriched diet has also been shown to increase the levels of antibodies to mbHsp65 (Maron et al., 2002). Recently, microbial Hsp60/65 (*E. coli* GroEL, cHsp60 and mbHsp65) antibodies have been shown to bind to certain hHsp60 epitopes, which are detected in apparently normal arterial walls but even more abundantly in atherosclerotic arterial walls (Perschinka et al., 2003). Interestingly, elevated mHsp60 antibody levels were seen in mice primarily infected with *C. pneumoniae* and then challenged with either *C. pneumoniae* or mock (cell remnants), which suggests that the primary *C. pneumoniae* infection had primed the mice for the booster effect of the infection or the mock inoculation. High mHsp60 IgG levels were demonstrated in mice with high *C. pneumoniae* Hsp60 IgG levels, but not all of the mice with high cHsp60 IgG levels exhibited high mHsp60 levels. In C57BL/6J mice, too, the mHsp60-reactive IgG antibody levels were elevated after the second exposure to *C. pneumoniae*, regardless of diet, suggesting that the first infection had primed the mice to elicit a response to the host Hsp60 protein. Compared to the transient increase in mHsp60 antibodies induced by the cholesterol-enriched diet alone, the autoantibody response was prolonged when induced by *C. pneumoniae* infection. In the present study, C57BL/6J mice did not have equally high levels of mHsp60 IgG antibodies as NIH/S mice. Indeed, C57BL/6J has been shown to be a low responder to chlamydial Hsp60 and 70 proteins (Zhong and Brunham, 1992). Repeated microbial exposures starting early in life are suggested to be the reason for the generation of antibodies capable of reacting with self-epitopes in Hsps. In pathogen-free animals, self-reactive Hsp antibodies do not naturally occur (Grandia et al., 1991).

5.4 Atherosclerosis in the chronic *C. pneumoniae* infection model of C57BL/6J mice

The C57BL/6J mice repeatedly challenged with *C. pneumoniae* were chronically infected with *C. pneumoniae*, as shown by their persistent lung inflammation and the PCR positivity of different tissues for *C. pneumoniae*. The early lipid lesions, which were scattered focally in the subendothelium at the base of valve cusps in C57BL/6J mice, were significantly increased by *C. pneumoniae* infections. Indeed, lipid lesions of this kind bear some similarity to the earliest stages of atherosclerotic lesion development in human subjects (Ross, 1999). Previous mouse studies have shown that *C. pneumoniae* infection leads to accelerated atherosclerosis in C57BL/6J mice only if the mice are hypercholesterolemic, and further, only if the hypercholesterolemia has been inititated prior or simultaneously to the first *C. pneumoniae* challenge (Moazed et al., 1997; Blessing et al., 2000; Blessing et al., 2001; Blessing et al., 2002a). In the above studies, however, the low-fat diet with only cholesterol supplementation was not studied. In the present study, the feeding of mice with a 0.2 % cholesterol-supplemented diet was initiated two weeks prior to the first *C. pneumoniae* challenge, but the cholesterol homeostasis in circulation was not markedly affected, nor was hypercholesterolemia induced with this diet. The distribution of cholesterol between the lipoprotein subclasses (i.e. levels of VLDL, LDL, and HDL) was not studied. If there had been even minor changes, the data might have produced relevant information. In the cholesterol-supplemented diet, the total fat content was identical to that of the regular chow diet (4 %) used. Further, the diet did not contain a cholate supplement, which contributes to the elevation of cholesterol levels in the circulation of C57BL/6J mice, and which was included in the diets of the previous studies with hypercholesterolemic mice (Vergnes et al., 2003). It is essential in C57BL/6 mice to suppress hepatic sterol-7-hydroxylase (CYP7A1) by cholate, the major regulator of the synthesis of bile acid from cholesterol (Dueland et al., 1993), whose overexpression diminishes the progression of atherosclerosis induced by an atherogenic diet (Miyake et al., 2002). Thus, the present study suggests that a cholesterol increment in the diet, although not associated with changes in plasma total cholesterol, is needed for the accelerated development induced by the infectious burden (i.e. persistent *C. pneumoniae* infection here). The cholesterol and cholate components of an atherogenic diet have been shown in C57BL/6J mice to induce the expression of genes involved in the proatherogenic inflammatory responses, such as acute inflammatory response and fibrinogenesis (Vergnes et al., 2003). Interestingly, dietary cholesterol, without cholate, is critical in the activation of acute inflammatory genes in C57BL/6J mice (Vergnes et al., 2003). As was shown in the present study, the dietary cholesterol-supplement exaggerated the *C. pneumoniae* pulmonary infection in C57BL/6J mice. The mechanisms leading to this remain to be studied.

The effects of repeated *C. pneumoniae* infections on serum lipid levels were, in agreement with the previous studies, virtually absent in C57BL/6J mice on both diets at the time points studied several weeks after the *C. pneumoniae* challenges (Blessing et al., 2001). In the acute phase of *C. pneumoniae* infection, changes in lipid levels are present in NIH/S mice, and may thus also be present in C57BL/6J mice (Tiirola et al., 2002). Also in agreement with the previous studies is the finding that *C.pneumoniae* infections do not induce atherosclerosis in C57BL/6J mice without a modification of diet (Moazed

et al., 1999; Blessing et al., 2000). The development of autoimmunity to mHsp60, as previously proposed by Wick and coworkers, may have participated in the development of early atherosclerotic lesions.

5.5 Antichlamydial effect of simvastatin

In a wider sense, statins, i.e. the lipid-lowering inhibitors of cholesterol biosynthesis, may act as inhibitors of intracellular signaling in eucaryotic cells and in the procaryotes that have HMG-CoA reductase of their own. The present study showed that the treatment of mice with lipophilic simvastatin at a dose corresponding to the therapeutic dose for treating hypercholesterolemia in humans (0.5 mg/kg) affected the course of acute *C. pneumoniae* infection by decreasing chlamydial counts and *C. pneumoniae* DNA in the lungs during the early phases of the infection. The present study does not allow interpretations of the long-term effects of statin treatment on the persistence of *C. pneumoniae* infection, which is considered important for the development of atherosclerosis.

The observed anti-chlamydial effect of simvastatin could have been mediated through a disturbance in the intracellular trafficking of cholesterol (Kempen et al., 1991; Keidar et al., 1994). Chlamydiae, and another gram-negative bacterium, *Salmonella*, which are inhibited by statin treatment (Kothe et al., 2000; Catron et al., 2004), do not have the capacity to synthesize cholesterol, but cholesterol is required for their successful multiplication in the intracellular compartment (Lange et al., 2000; Carabeo et al., 2003; Catron et al., 2004). Chlamydiae may use cholesterol of the infected cells, derived from either the extracellular space, like that derived from LDL uptake, or the intracellular cholesterol stores from *de novo* synthesis (Carabeo et al., 2003). An important step where statins may interfere with chlamydial infection is the chlamydial entry into cells, as in the case of HIV infection (del Real et al., 2004). Detergent-resistant lipid microdomains in the plasma membrane, caveolae, or lipid rafts are rich in cholesterol, and these domains have been shown to be important for the entry of several *Chlamydia* species and biovars, including *C. pneumoniae* (Stuart et al., 2003). The statin may have taken over numerous biochemical functions of host cell, which are important for chlamydial infection, through inhibition of isoprenoid biosynthesis (Kothe et al., 2000).

This inhibition may also be partly immune-mediated. In the present study, the pulmonary inflammatory response to *C. pneumoniae* infection was – opposite to most pre-existing literature reports - increased by the statin treatment of mice on both diets. The anti-inflammatory action that statins exhibit has been reported in several studies conducted in different experimental conditions. In one mouse model, oral administration of simvastatin to mice inhibited innate immunity by reducing the oedema (influx of polymorphonuclear leukocytes) of a carrageenan-induced inflammatory reaction in the mouse foot pad (Sparrow et al., 2001). In another study, atorvastatin diminished histological changes in Th1-mediated central nervous system autoimmune disease by promoting Th2 type immunity and resting Th1 type immunity, which resulted in decreased IFN-γ secretion among other things (Youssef et al., 2002). Airway inflammation in an OVA-induced asthma model has also been reported to be decreased by simvastatin treatment (McKay et al., 2004). Another group has further demonstrated that pravastatin treatment

of OVA-challenged mice reduces total cell counts and eosinophils, but not neutrophils, lymphocytes, or macrophages in the lungs (Yeh and Huang, 2004). A common feature of all these studies is the use of noninfectious inflammatory stimuli. The present study is the first to focus on the effect of statin treatment on immunological parameters after an active infection caused by a live, replicating pathogen *in vivo* under controlled experimental conditions. Kiener *et al* previously pointed out the proinflammatory effects of statins. Pretreatment of mice with simvastatin before an inflammatory challenge with thioglycollate transiently increased the influx of inflammatory cells into the peritoneal cavity (Kiener et al., 2001). In the present study, the increased inflammatory response may reflect the intensified reaction, which would be beneficial in defense against the invading pathogen. Statins also induce apoptosis, and thus, in simvastatin treated mice, *C. pneumoniae* liberated from infected cells after apoptosis could have been more extensively exposed to immune defense. In general, SAA levels parallel those of CRP. SAA binds to HDL and may affect cholesterol metabolism and also contribute to the oxidation of LDL (reviewed in (Gabay and Kushner, 1999)). In the present study, both SAA and LBP levels increased moderately and returned to baseline, but simvastatin therapy did not modulate this acute phase response during primary infection.

In mice, a cholesterol-lowering effect of statins has been achieved with high treatment doses even in individuals lacking functional low-density lipoprotein receptors (Bisgaier et al., 1997). However, with the simvastatin dose used in the present study, no decrease in the total cholesterol or triglyceride levels either in the mice on a regular diet or in those on a high-fat diet was seen. The response to a high-fat diet was as expected, and elevated levels of serum total cholesterol and triglycerides were observed.

6 Conclusions and future prospects

The present study highlighted the importance of the appropriate performance of the infectious challenge by comparing the different anesthetic agents used to inoculate animals. It also showed that the host cell debris in *C. pneumoniae* inoculum may possibly have some contribution to the infection model: inoculation with host cell debris alone induced a weak inflammatory response and reactivated culture-negative *C. pneumoniae* infection in the lungs.

When investigating the lung histopathology of mice infected via different routes, it turned out that either *C. pneumoniae* was able to disseminate from the gastrointestinal tract into the lungs through circulation and/or lymphatics or the lymphocytes that had encountered gastrointestinal tract-derived *C. pneumoniae* were homing into the lungs.

When two different Finnish *C. pneumoniae* isolates, K6 and K7, were used in parallel in the present study, the courses of pulmonary infection differed slightly.

The present results further indicated that repeated inoculations led to the development of a Hsp60 autoimmune response, and further, without overt hypercholesterolemia, repeated *C. pneumoniae* infections enhanced the development of early atherosclerosis lesions, offering an experimental model to study chronic *C. pneumoniae* infection and early atherosclerotic events. It was also shown that cholesterol feeding alone transiently increased Hsp60 autoimmune antibodies, but when it was combined with repeated *C. pneumoniae* infections, a stronger response developed than with either *C. pneumoniae* or cholesterol feeding alone. Thus, even a small increment of dietary cholesterol alone may induce the formation of antibodies to mHsp60, but for persistent Hsp60 autoimmunity, the presence of cHsp60 in the stressed cells may possibly also be needed. Further, the chronic pulmonary inflammation due to *C. pneumoniae* was shown to be exaggerated in mice on a cholesterol-supplemented diet.

In the present study, treatment of mice with simvastatin decreased chlamydial growth, as has been seen in previous *in vitro* studies using cerivastatin. The statin used in the present study is lipophilic, like cerivastatin, and thus able to access cells in which *C. pneumoniae* is able to multiply. Simvastatin treatment also exerted an immunomodulatory effect, but an unexpected one, by increasing the amount of inflammatory cells in lungs.

Further questions arise from the present findings. A cellular preparation consisting only of host cells was shown to reactivate quiescent *C. pneumoniae* infection in the lungs.

We can speculate that other microbial infections or any immunological stimulus might also have a similar reactivating effect on chronic infection, thus accelerating the development of chronic sequelae such as atherosclerosis. Further animal studies using other microbial challenges after established chronic *C. pneumoniae* infection are needed to find answers to these questions.

The experimental *C. pneumoniae* mouse models developed to study infection-induced atherosclerosis are still poorly characterized. The application of genomics and proteomics to study microbe-host interaction during the development of chronic infection in mouse models will provide valuable information and allow comparisons with *C. pneumoniae* infection in human.

The interesting finding on the antichlamydial effect of simvastatin in acute infection should be expanded to a chronic infection model as well as to studies on the markers of systemic inflammation during chronic *C. pneumoniae* infection. These studies will hopefully provide us with the information of whether the beneficial effect of statins on cardiovascular diseases may, at least partly, be explained by their antichlamydial (and more generally, antimicrobial) effects.

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